DEVELOPMENT, CHARACTERIZATION AND EVALUATION OF NTPDase-MODIFIED POLYMER FOR INHIBITING PLATELET DEPOSITION

By

VIGNESH MUTHUVIJAYAN

Bachelor of Technology
A. C. Tech, Anna University
Chennai, Tamil Nadu, India
2002

Master of Science
University of Maryland, Baltimore County
Baltimore, MD
2004

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Dissertation Approved:

Dr. Randy S. Lewis

Dissertation Adviser

Dr. Sundararajan V. Madihally

Dr. Gary L. Foutch

Dr. Karen A. High

Dr. Warren T. Ford

Dr. A. Gordon Emslie

Dean of the Graduate College

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NOMENCLATURE

5-HT	Serotonin
ADA	American Dental Association
ADP	Adenosine diphosphate
ADPase/CD39	Endothelial ecto-adenosine phosphatase
AFM	Atomic force microscope
AlbSH	Albumin
AlbSNO	S-nitroso albumin
AMP	Adenosine monophosphate
ASTM	American Society of Testing and Materials International
ATIII	Antithrombin III
ATP	Adenosine triphosphate
ATR-FTIR	Attenuated Total Reflection Fourier Transform Infrared
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
cGMP	3, 5-cyclic guanosine monophosphate
CySH	Cysteine
CySNO	Nitrosated cysteine
EDC	1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride

EDRF	Endothelium derived relaxing factor
eNOS/NOS III	Endothelial nitric oxide synthase
FDA	Food and Drug Administration
GP	Glycoprotein
GTP	Guanosine triphosphate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMWK	High molecular weight kininogen
iNOS/NOS II	Inducible nitric oxide synthase
ISO	International Standards Organization
MES	2-(N-morpholino) ethanesulfonic acid
MG	Malachite green assay reagent
NIH	National Institutes of Health
nNOS/NOS I	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
NTPDase	Nucleoside triphosphate diphosphohydrolase
PAI	Plasminogen activator inhibitor
PBS	Phosphate buffered saline
PEO	Polyethylene oxide
PET	Polyethylene terephthalate
PET-COOH	Carboxylated PET
PET-COOH-Cys	Cysteine-modified carboxylated PET
PET-COOH-NTPDase	NTPDase-modified carboxylated PET

PET-NH_2	Aminolyzed PET
PET-NH_2-NTPDase	NTPDase-modified aminolyzed PET
PF4	Platelet factor 4
PGI_2	Prostacyclin
Pi	Inorganic phosphate
PK	Prekallikrein
PMEA	Poly-2-methoxy ethylacrylate
PMMA	Polymethyl methacrylate
PRP	Platelet rich plasma
PVA	Polyvinyl alcohol
RBC	Red blood cells
RSH	Sulfhydryl amino acids
RSNO	S-nitrosothiols
SDS	n-Sodium dodecyl sulfate
SEM	Scanning electron microscope
sGC	Soluble guanylate cyclase
SMA	Surface modifying additives
TFPI	Tissue factor pathway inhibitor
TM	Thrombomodulin
t-PA	Tissue plasminogen activator
TXA_2	Thromboxane A_2
u-PA	Urokinase plasminogen activator
vWF	von Willebrand factor

WBC	White blood cells
α_2-MG	α_2-macroglobulin

CHAPTER 1

INTRODUCTION

According to the definition provided by Clemson University Advisory Board for Biomaterials at the 6th Annual International Biomaterial Symposium (1974), "a biomaterial is a systemically and pharmacologically inert substance designed for implantation within or incorporation with living systems". Biomaterials include any material, such as metals, ceramics, carbons, composites and polymers, which intermittently or continuously comes in contact with biological systems. Biomaterials have a vast array of applications ranging from catheters, probes, sutures and bone plates to vascular grafts, heart valve prostheses, cardiac pacemakers, joint and limb replacements, renal dialyzers and blood oxygenators. With such a wide range of applications, design and development of compatible biomaterials is critical for construction of both extracorporeal devices and implants. The first reported use of "biomaterials" was in 1759, when Hallowell repaired an injured artery with a wooden peg and twisted thread (Wesolowski and Dennis 1963). However, use of biomaterials in surgery was not successful until the development of aseptic surgical techniques by Dr. J. Lister in the 1860s, due to infections (Park 1984). As infections were drastically reduced with widespread implementation of aseptic surgical procedures, the effect of the properties of biomaterials used in the success of implantation was recognized (Friedman *et al.* 1994).

With this understanding, the interest in developing effective biomaterials for successful implantation grew. Early implants were largely applied to healing broken bones and joints. In the 1900s, biomaterials were primarily chosen based on their mechanical strength and they usually corroded rapidly (Woo *et al.* 2004). In the 1930s, stainless steel and cobalt-chromium based alloys were introduced in fracture repair and joint replacement surgeries (Park 1984). In 1946, the Judet brothers from France developed the first biomechanically designed hip prosthesis using polymethyl methacrylate (PMMA) (Park 1984). At around the same time, PMMA was also used for corneal replacement (Park 1984). After these developments, use of plastics and polymers as biomaterials grew exponentially.

With the knowledge gained through the past experiences, the first biomaterials were developed. These were mostly commonly available materials. For example, cellulose acetate, a commodity plastic, was used for dialysis tubing; Dacron, a textile polymer, was used for vascular grafts and commercial grade polyurethanes were used for artificial heart parts (Woo *et al.* 2004). Although these materials were partly effective, they also introduced serious complications due to inflammatory reactions. For instance, dialysis tubing activated platelets and the complement system; vascular grafts made from Dacron could be used only when the diameter was larger than 6 mm, otherwise the graft was clogged due to blood coagulation and blood clots formed because of blood-biomaterial interaction in artificial hearts posed serious possibility of stroke or other complications. The limitations experienced were due to lack of biocompatibility of the materials used.

Biocompatibility is defined as “the ability of a material to perform with an appropriate host response in a specific application” (Williams 1987). This would include the physical properties of the biomaterial and the biological reaction occurring due to the interaction between the biomaterial and the host. Biocompatibility of materials coming in direct contact with blood is called haemocompatibility. Lack of haemocompatibility causes serious complications like platelet adhesion and aggregation, thrombus formation and shedding of emboli (Ratner 1996; Mowery *et al.* 2000; Schoenfisch *et al.* 2000). Such complications not only compromise the device function, but also cause serious side effects like severe bleeding, stroke, ischemia, vasospasm, hypertension and inflammation, all of which can be life threatening in patients (Vallance 1989; Traub and Berk 1998).

Most of the blood-contacting materials used today are not completely haemocompatible. To overcome the limitations in haemocompatibility of biomaterials, thrombin and platelet antagonists like heparin and prostacyclin are added to blood-based perfusates to reduce thrombogenicity. However, the drawback is that loss of heparin activity over time results in loss of haemocompatibility (Larsson *et al.* 1987). Also, antithrombogenicity induced by heparin and prostacyclin is not site-specific due to the deactivation of coagulation factors in the plasma (Larsson *et al.* 1987). Hence, improving the haemocompatibility of biomaterials is a major concern. Two possible ways to improve haemocompatibility of blood-contacting surfaces are to develop new biomaterials with surfaces which are completely haemocompatible or modify the surface of currently available biomaterials. It is usually complicated to develop a new biomaterial with desired physical and biological properties. Hence, modifying surfaces of currently available biomaterials is a more viable solution.

Many surface modification techniques are currently used to improve haemocompatibility of biomaterials. One approach is to disguise the biomaterial surface in such a way that it is not recognized as a foreign body. One technique to achieve this is albumin passivation (Kottke-Marchant *et al.* 1989). It has been shown that platelet activation was reduced by pre-coating albumin to the surface of tubing in an extracorporeal system (Amiji *et al.* 1992). However, during prolonged exposure to blood, the albumin was replaced by other proteins (Vroman and Adams 1969; Vroman *et al.* 1980) and hence the material lost its haemocompatibility. Also, this technique doesn't prevent platelet activation in extracorporeal devices (Borgdorff *et al.* 1999).

Other techniques used for surface disguising are endothelial seeding (Herring *et al.* 1984) and biomembrane mimicry by coating with phospholipids such as phosphorylcholine (Durrani *et al.* 1986; Chapman and Lee 1987). However, these techniques change the surface properties and are effective only for medium periods of time (Lowe *et al.* 2005). Also, the time, money and energy required for preparing and storing these biomaterials is a serious limitation (Tanzi 2005). Another surface modification technique currently used is attaching hydrophilic chains, such as poly-2-methoxyethyl acrylate (PMEA) (Saito *et al.* 2000; Ninomiya *et al.* 2003) and polyethylene oxide (PEO) (Golander and Kiss 1988; Brinkman *et al.* 1989; Nojiri *et al.* 1990; Litauszki *et al.* 1997) to the surface. This minimizes adsorption and denaturation of proteins and blood cells, thereby improving haemocompatibility. However, the long chain polymers present on surface of the biomaterial can cause steric hindrance that would alter the biological property to the material.

Mixing synthetic materials with surface modifying additives (SMA) during production phase has been done to improve haemocompatibility (Tsai *et al.* 1994; Gu *et al.* 1998). However, clinical studies showed that only minor improvements were observed using SMA treated devices (van Oeveren 2005). Another commonly used technique to improve haemocompatibility is attaching biologically active compounds (Han *et al.* 1989; Seifert *et al.* 1997). This is achieved by grafting these molecules to the biomaterial and releasing it over a period of time. Some of the compounds currently used are heparin (Han *et al.* 1989), hirudin (Seifert *et al.* 1997) and NO-releasing compounds (Smith *et al.* 1996). However, the biomaterial loses haemocompatibility once the active molecule is completely released and hence prolonged haemocompatibility is not achieved. The other major problem associated with this technique is the difficulty to control the release rate. This could potentially lead to dangerously high levels of the compound in the blood stream.

As seen in Table 1.1, millions of medical devices use blood-contacting biomaterials. Improving haemocompatibility of the blood-contacting biomaterials will vastly improve the clinical application and save and improve the lives of millions of patients. As the market for biomaterials exceeds $100 billion (Peppas and Langer 1994; Ratner *et al.* 2004a), the economic impact will also be very significant. According to the American Heart Association, from 1979 to 2005, the total number of inpatient cardiovascular operations and procedures has increased 484 percent to 6,989,000 annually (Rosamond *et al.* 2008). The number of cardiac catheterizations has increased 342 percent (Rosamond *et al.* 2008). An estimated 1,322,000 cardiac catheterizations were performed in 2005 (Rosamond *et al.* 2008).

Table 1.1. Number of medical devices involving blood-contacting polymers in US per year (Global numbers are typically 2 – 3 times the US number) (Ratner *et al.* 2004b)

Medical Devices	Number/year
Vascular Graft	300,000
Catheter	200,000,000
Blood Bags	40,000,000
Heart Valve	100,000
Coronary Stents	1,500,000
Pacemaker	400,000
Heart-Lung Oxygenators	300,000
Renal Dialysis (number of patients, 2001)	320,000
Left Ventricular Assist Devices	100,000

Also, it is estimated that 469,000 coronary artery bypass procedures on 261,000 patients (Pleis and Lethbridge-Cejku 2006) and 699,000 open-heart surgery procedures (Rosamond *et al.* 2008), both using extracorporeal blood oxygenators, were performed. An estimated 1,271,000 angioplasty procedures were performed (Rosamond *et al.* 2008). The number of implantable defibrillators, pacemakers and heart valves included 91,000; 180,000; and 106,000 respectively (Rosamond *et al.* 2008). These statistics represent only the cardiac applications of biomaterials. The numbers will be a lot higher if all applications using blood-contacting devices were considered.

This research focuses on modifying the surface of a polymeric biomaterial to improve the haemocompatibility. Polymeric biomaterials were chosen to be explored because of the wide range of blood-contact applications. Synthetic polymers have been used in catheters, blood bags, dressings, implants and extracorporeal devices (Lee *et al.* 2003). The main advantage of polymeric biomaterials is the ease of producing different shapes with desired mechanical and physical properties. Polymeric biomaterials are also cost effective. These advantages make polymers a viable option.

Polyethylene terephthalate (PET) was chosen as the model polymer. PET is a linear, aromatic polyester shown to have the desirable mechanical strength and durability for biomedical applications (Bide *et al.* 2006; Nissen *et al.* 2008). PET also has a wide range of medical applications which include vascular prostheses (Vinard *et al.* 1988; Kottke-Marchant *et al.* 1989; Haulon *et al.* 2003; Blanchemain *et al.* 2007a; Blanchemain *et al.* 2007b), heart valve sewing cuffs (Tweden *et al.* 1997; Illingworth *et al.* 1998; Jin *et al.* 2007), implantable sutures (Homsy *et al.* 1968; 21CFR878.5000 2008), and surgical mesh (Zieren *et al.* 2004; Bracco *et al.* 2005).

With several of the PET applications involving contact with blood, PET was a suitable model polymer to be explored for this research. However, PET is an inert polymer and lacks active functional groups on the surface to attach biologically active molecules. Hence, surface functionalization of polyethylene terephthalate is necessary for immobilizing biomolecules which will improve haemocompatibility.

The goal of this work is to improve the haemocompatibility of PET surfaces by identifying and utilizing a naturally occurring platelet inhibition mechanism. In nature, there are multiple anti-coagulant pathways to regulate blood clotting. One such mechanism is adenosine diphosphate (ADP) scavenging. Platelets exposed to foreign surfaces get activated and secrete ADP. ADP leads to further irreversible platelet aggregation. Scavenging the ADP secreted will inhibit irreversible platelet aggregation. In nature, ADP induced platelet aggregation is counteracted by an enzyme called nucleoside triphosphate diphosphohydrolase (NTPDase). NTPDase rapidly metabolizes ADP to release adenosine monophosphate (AMP) and inorganic phosphate (Pi), resulting in the platelets to return to the resting state (Marcus *et al.* 1997). Bakker et al. had used NTPDase as the biologically active compound for inhibiting platelet deposition (Bakker *et al.* 1991; van der Lei *et al.* 1992). Their results show that NTPDase coating on polyurethane vascular prostheses improves haemocompatibility. However, for the case of NTPDase coated polyurethane, rapid release of the non-specifically bound NTPDase could result in undesired effects. Also, once the coated NTPDase is washed off, the polymer would lose the haemocompatibility. Biomaterials with covalently attached NTPDase have shown enhanced platelet inhibition when *in vivo* studies were performed by insertion into a dog's femoral vein (Marconi *et al.* 1983). Marconi et al. immobilized

NTPDase to hydrolyzed PET, despite hydrolyzed PET showing loss of mechanical strength due to hydrolysis of PET (Ellison *et al.* 1982; Sanders and Zeronian 1982; Kao *et al.* 1998; Liu *et al.* 2005). Based on these studies, effective NTPDase immobilization to mechanically stable PET would potentially enhance haemocompatibility.

Another thromboregulatory mechanism that inhibits platelet adhesion involves the release of endothelium derived relaxing factor (EDRF) or nitric oxide (NO). Studies have shown that NO inhibits platelet adhesion by a cGMP dependent mechanism (Mellion *et al.* 1981; Konishi *et al.* 1996). Some studies had been performed to improve haemocompatibility by attaching NO-releasing compounds to polymer surfaces (Saavedra *et al.* 1996; Smith *et al.* 1996). However, attaching NO-releasing compounds has a number of limitations including superoxide formation, interactions with iron and exhaustion of NO. It has been established that cysteine molecules attached to biopolymer surfaces utilize endogenous NO and inhibit platelet adhesion by up to 67% (Duan and Lewis 2002). This eliminates the limitations involved in attaching NO-releasing compounds. Furthermore, a study has shown that NTPDase enhanced the platelet inhibition effect of NO in an additive dose-dependent manner (Ramamurthi *et al.* 2001). Hence, attaching both NTPDase and cysteine to PET surface will exhibit enhanced platelet inhibition.

The objectives for this project include modifying PET surfaces to introduce reactive functional groups, successfully immobilizing NTPDase, characterizing the modified polymer to understand surface and bulk properties, studying the enzyme kinetics of immobilized NTPDase, demonstrating improved haemocompatibility and

immobilizing NTPDase and cysteine together on PET surface. These objectives are outlined in more detail below.

1.1 Objective #1: Analysis of functionalized PET

As mentioned earlier, PET is an inert polymer and lacks active functional groups on the surface. Hence, surface functionalization of polyethylene terephthalate is necessary for immobilizing biomolecules. Currently, many techniques such as hydrolysis (Ellison *et al.* 1982; Kao *et al.* 1998; Bide *et al.* 2006), reduction (Bùi *et al.* 1993), glycolysis (Fadeev and McCarthy 1998), aminolysis (Ellison *et al.* 1982; Avny and Rebenfeld 1986; Duan and Lewis 2002; Gappa-Fahlenkamp and Lewis 2005; Bide *et al.* 2006; Liu *et al.* 2008), amination (Nissen *et al.* 2008) and carboxylation (Yang *et al.* 2000) have been utilized. In this work, aminolysis, hydrolysis and carboxylation were performed to introduce functional groups on a PET surface. Surface characteristics of the modified PET were studied using a scanning electron microscope (SEM) and an atomic force microscope (AFM). To understand the bulk properties of the modified polymer, tensile strength was studied. NTPDase was covalently attached to the modified polymers. The attachment was verified by studying the activity of NTPDase.

1.2 Objective #2: Kinetic analysis of immobilized NTPDase

Immobilized NTPDase kinetics were studied and compared with free NTPDase kinetics. Immobilized NTPDase kinetics were modeled. The stability and the

effectiveness of the attachment were also analyzed. The effects of storage on NTPDase-modified PET was also analyzed.

1.3 Objective #3: In vitro studies of NTPDase-modified PET

For analyzing the haemocompatibility of NTPDase-modified polymer, protein adsorption *in vitro* studies were performed. For this purpose, adsorption of bovine serum albumin on the modified polymer was measured and compared with unmodified polymer. Platelet-rich plasma studies were performed to analyze platelet deposition on the modified surfaces. Whole blood analysis was also performed to verify haemocompatibility. These results were compared to that of unmodified polymer.

1.4 Objective #4: Immobilization of both NTPDase and cysteine

NTPDase and cysteine were immobilized on the surface of carboxylated PET. The attachment was verified by studying the activity of NTPDase and measuring the concentration of cysteine on the polymer surface. Stability of the attachment was studied.

While developing a material for biomedical applications, it is important to understand the biocompatibility of the material. It is also critical to evaluate the surface and the bulk properties of the material, as these properties play a vital role in determining the applications in which the material can be used. However most of the research performed in developing haemocompatible biomaterials does not analyze the surface and the bulk properties. Without this information, it is very difficult to evaluate the

applications of biomaterials. Although there are many studies involving improving haemocompatibility of biomaterials, this work is unique because it discusses both aspects involved in developing a useful biomedical material, namely, physical properties of the modified polymer and ability to immobilize biologically active molecules to the modified surface. In this research, a haemocompatible polymer with desired surface and bulk properties was developed. This study also provides further insight into the potential of utilizing ADP scavenging as a technique to improve haemocompatibility. This study also explores the potential of utilizing both NTPDase and cysteine simultaneously to possibly provide enhanced haemocompatibility. As NTPDase and cysteine inhibit platelet adhesion via separate mechanisms, immobilizing NTPDase cysteine would utilize two different endogenous platelet inhibition pathways simultaneously. Such a method would be a significant improvement over the current technologies.

CHAPTER 2

LITERATURE REVIEW

For developing a biomaterial that utilizes naturally occurring anti-coagulant pathways to improve haemocompatibility, understanding the blood coagulation mechanism and the anti-coagulant pathways is critical. Furthermore, in-depth knowledge of desirable properties of biomaterials is also vital for developing a viable biopolymer. This chapter reviews the mechanisms and the components involved in the blood interaction with biopolymer surfaces. The current techniques that are used for improving haemocompatibility and their limitations are also discussed. Functionality of NTPDase in regulating blood coagulation and its potential in improving haemocompatibility of biomaterials are examined thoroughly.

2.1 Constituents of Blood

Blood is a specialized bodily fluid that acts as a vehicle of transportation mediating the cellular interactions and physiological mechanisms. Blood constitutes about 7% of human body weight with an average volume of around 5 liters (Alberts 2005). Blood contains various types of cells suspended in plasma (Beutler and Williams 1995). The formed elements of blood are erythrocytes or red blood cells (RBC),

leukocytes or white blood cells (WBC) and platelets. These cells constitute 45% of the whole blood volume and the plasma constitutes the remaining 55%.

2.1.1 Plasma

A normal adult has an average of 25 to 45 ml of plasma per kg of body weight (Berne and Levy 1993). Plasma is a complex aqueous medium in which innumerable substances are dissolved (Ratnoff 1983). Some of the substances dissolved in plasma include oxygen, carbon dioxide, nitrogen, electrolytes, proteins, lipids, carbohydrates, amino acids, vitamins, hormones and nitrogenous breakdown products of metabolism. The concentration of these substances varies with diet, metabolism and the levels of hormones and vitamins. Usually, many homeostatic mechanisms help in maintaining the composition of blood in a biologically safe and useful range.

The charged components present in the plasma help in maintaining the osmolarity and pH of blood within physiological limits (Ratnoff 1983). The primary inorganic cation in plasma is sodium. Plasma also contains lesser quantities of ionic potassium, calcium and magnesium (Ratnoff 1983). The primary anion in plasma is chloride. Plasma also contains bicarbonate, plasma protein, phosphate, sulfate and organic acids (Ratnoff 1983). Highly structured metabolic and physiological processes regulate the concentration of ions present in the plasma to maintain the normal composition.

Plasma also contains hundreds of dissolved proteins. Total protein concentration in plasma is approximately 7 g/dl (Berne and Levy 1993). Most the proteins are of two types, namely, albumin and immunoglobulins. Albumin, which is synthesized in the liver

by hepatic parenchymal cells, is present at a concentration of 4 g/dl (Fournier 1999). Albumin plays a key role in providing colloid osmotic or oncotic pressure as it doesn't diffuse easily through intact vascular endothelium. This helps in regulating the passage of water and diffusible solutes through the capillary (Ratnoff 1983). Albumin is also a carrier for substances that are adsorbed to it.

Immunoglobulins or antibodies constitute the second highest concentration in blood plasma. These are synthesized by the plasma cells in the lymphoid organs (Ratnoff 1983). Antibodies play a vital role in defense against infections. On exposure to antigens, which are usually stimulants foreign to the normal body, lymphocytes get stimulated. This results in formation of specific antibodies. Immunoglobulins are classified into five groups based on the amino acid composition, structure and functions. They are IgG, IgM, IgA, IgD and IgE. IgG antibodies are found in all body fluids. They are the smallest but most common antibody (75% to 80%) of all the antibodies in the body (Pier *et al.* 2004). IgG antibodies are very important in fighting bacterial and viral infections. IgG antibodies are the only type of antibody that can cross the placenta in a pregnant woman to help protect her baby (Pier *et al.* 2004).

Plasma also contains clotting factors required for blood coagulation, such as fibrinogen, the most abundant; complement, a group of proteins involved in immune responses; enzymes, enzyme precursors and enzyme inhibitors; specific carriers of constituents like iron and copper; and scavengers of agents inadvertently released in to the plasma (Ratnoff 1983). Plasma lipids such as triglycerides, phospholipids, cholesterol and fatty acids are transported as complexes with plasma proteins (Ratnoff 1983).

2.1.2 Red Blood Cells

Red blood cells or erythrocytes contribute to the majority of the cellular composition of blood. RBCs occupy 48% of the total volume of blood in men and 42% in women (Ratnoff 1983). The percent of RBC in blood is called as hematocrit. The red blood cell count ranges between 4-6 million/μl (Ratnoff 1983). Red blood cells are continuously produced in our bone marrow from stem cells. Red blood cells are devoid of a nucleus, as they lose their nuclei before entering into the blood stream. As seen in Figure 2.1a, red blood cells are shaped like a biconcave lens. Vitamin B12, folic acid and iron are required for effective maturation of red blood cells. Iron is required for formation of hemoglobin, the primary constituent of the cytoplasm of red blood cells (Ratnoff 1983). Hemoglobin is produced in the red blood cell precursors inside the bone marrow. Hemoglobin is a complex protein containing four polypeptide “globin” subunits attached to a prosthetic group consisting of tetrapyrrole heme. Each heme unit surrounds an atom or iron. Atmospheric oxygen, diffusing into the red blood cells, attach to this iron atom. Oxygen is then released to the tissues in the peripheral capillaries. Hemoglobin is also involved in transport of some carbon dioxide attached to the globin portion (Ratnoff 1983). Reduced levels of red blood cells in the blood stream is called anemia. This occurs due to reduced synthesis of red blood cells or premature destruction or loss due to hemorrhage. Reduced synthesis could occur due to deficiencies in vitamin B12 or iron, which are required for formation of hemoglobin. Life span of red blood cells is normally about 120 days (Ratnoff 1983). This may be shortened due to damage caused by antibodies resulting in a process called hemolysis.

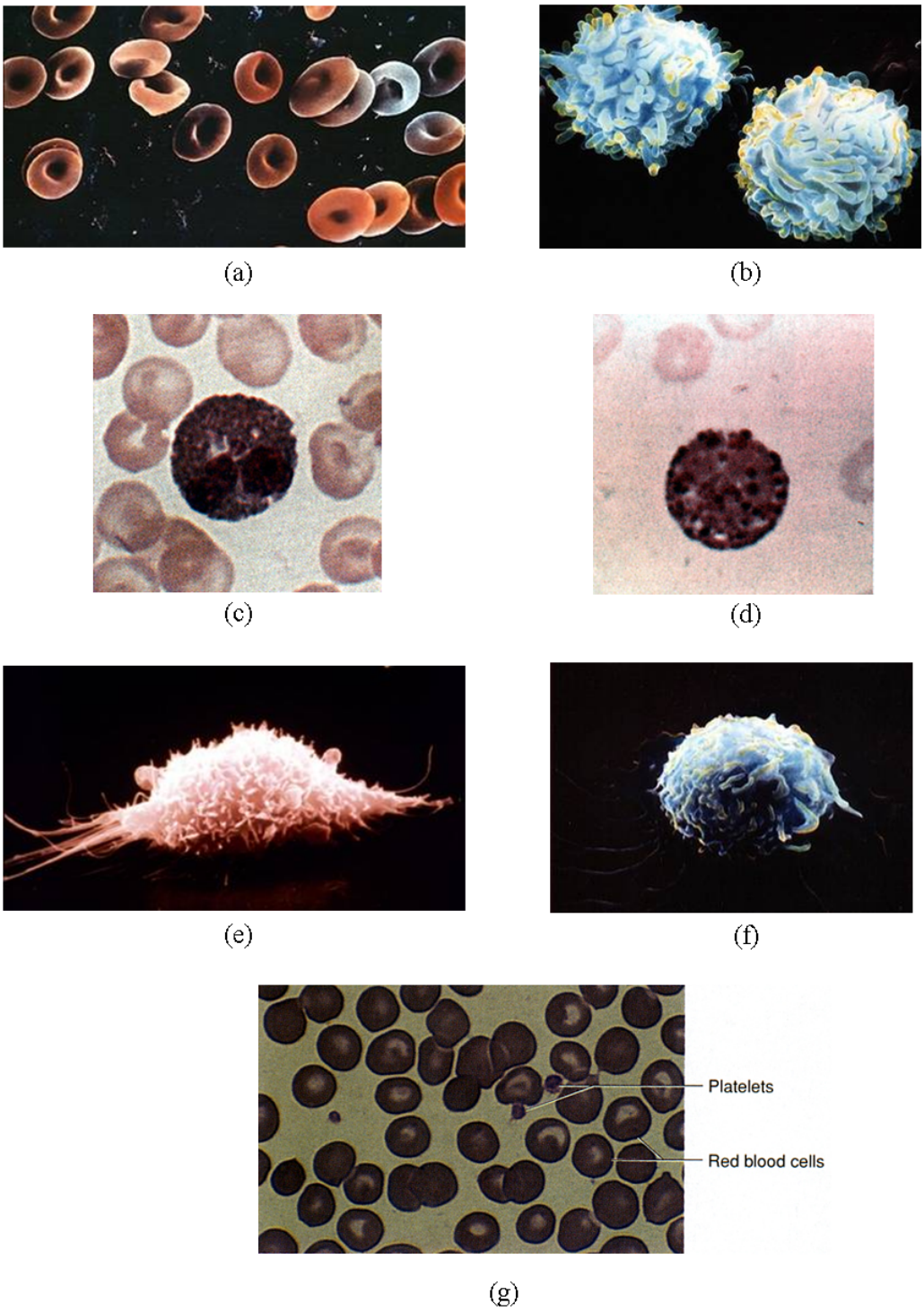


Figure 2.1. Cellular Components of Blood. (a) Erythrocytes or Red Blood Cells; (b) Neutrophils; (c) Eosinophils; (d) Basophils; (e) Monocytes; (f) Lymphocytes; (g) Platelets. Images were obtained from The Merck Manuals: Online Medical Library (Frenkel 2006).

2.1.3 White Blood Cells

White blood cells or leukocytes are a small portion of blood cellular constituents. Normal WBC count in human blood is between 4000-10,000 cells/μl. White blood cells are responsible primarily for defending the body against infection. White blood cells can be categorized into five classes, namely, neutrophils, eosinophils, basophils, monocytes and lymphocytes. Neutrophils (Figure 2.1b) are the most common type of leukocytes. Neutrophils protect the body against infections by killing and ingesting bacteria and fungi and ingesting foreign materials (Berne and Levy 1993; Beutler and Williams 1995; Frenkel 2006). Eosinophils (Figure 2.1c) are involved in allergic responses. They are also responsible for killing parasites and destroying cancer cells (Ratnoff 1983; Frenkel 2006). Basophils (Figure 2.1d) are also involved in allergic responses (Ratnoff 1983; Frenkel 2006). Neutrophils, eosinophils and basophils are collectively known as the granulocytes. Monocytes (Figure 2.1b) are the largest form of leukocytes with an average diameter of 15 to 20 μm (Ratnoff 1983). Monocytes ingest dead or damaged cells and help defend against many infectious organisms. Lymphocytes are a heterogeneous group of cells with large nuclei. Three types of lymphocytes have been identified based on differences in function. They are B-cells, T-cells and null cells. On stimulation by antigens, B-cells transform to produce specific immunoglobulin antibodies (Ratnoff 1983). T-cells help protect against viral infections and can detect and destroy some cancer cells. Null cells are also called as killer cells. They are capable of destroying tissue cells that are coated with antibody.

Some white blood cells flow smoothly through the bloodstream, but many adhere to blood vessel walls or even penetrate the vessel walls to enter other tissues. When white blood cells reach the site of an infection or other problem, they release substances that attract more white blood cells. The white blood cells function like an army, dispersed throughout the body but ready at a moment's notice to gather and fight off an invading organism. White blood cells accomplish this by engulfing and digesting organisms and by producing antibodies that attach to organisms so that they can be more easily destroyed.

2.1.4 Platelets

Platelets are anuclear disk-shaped cells found in the blood plasma (Figure 2.1g). They have an average diameter of 3-4 μm, a thickness ~1 μm and an average volume of around 10 μm^3 (Frojmovic and Panjwani 1976). Platelets are produced by megakaryotes in the bone marrow and have a life span of 8 to 12 days in humans (Ratnoff 1983). Platelets circulate at a average count of 250,000 cells per microliter of whole blood (Hanson 2004). The primary function of platelets is control of bleeding by causing blood coagulation. The mechanism of blood coagulation is discussed in detail in the next section. The organelles and components present in platelets (Figure 2.2) that are involved in blood coagulation are explained here.

Two glycoproteins on the platelet membrane, namely, glycoprotein (GP) IIb/IIIa and GP Ib-IX, act as principal receptors. GP IIb/IIIa receptor binds strongly to fibrinogen (Sims *et al.* 1991) and von Willebrand factor (vWF) (Savage *et al.* 1996) after platelet

activation leading to platelet aggregation. Platelet membrane phospholipids also play a role in platelet activation and clot formation. Actin microfilament present in the platelet cytoskeleton is responsible for developing filopodia, a long spike protruding from the growing tip on a cell, via polymerization and phosphorylation during activation (Fox and Phillips 1982). Microtubules in the cytoskeleton reassemble during platelet activation and cause the platelet shape change from discoid to round (Fox *et al.* 1993). The granular compartment and the membrane of α-granules store several important proteins involved in blood coagulation, including platelet factor 4 (Harrison *et al.* 1990), vWF, fibronectin, fibrinogen (Wencel-Drake *et al.* 1985), coagulation factor V (Hayward *et al.* 1995) and GP IIb/IIIa (Cramer *et al.* 1990). Dense granules contain adenosine diphosphate (ADP), adenosine triphosphate (ATP), Ca^{2+} and serotonin (5-HT). ADP acts as a trigger for platelet aggregation and calcium ions are critical for platelet functions as most enzymatic functions are calcium-dependent. Lysosomal granules are released slowly and incompletely, suggesting they play a greater role in anticoagulation (White 1993).

2.2 Blood Coagulation

Blood coagulation is an important defense mechanism to prevent excessive bleeding. It is also known as hemostasis. Hemostasis proceeds in two phases: primary and secondary hemostasis. Primary hemostasis is characterized by platelet adhesion to the damaged blood vessel, platelet activation due to agonists, and platelet aggregation via fibrinogen. Primary hemostasis is short lived. If flow is allowed to increase, the platelet aggregate could be sheared from the injured surface.

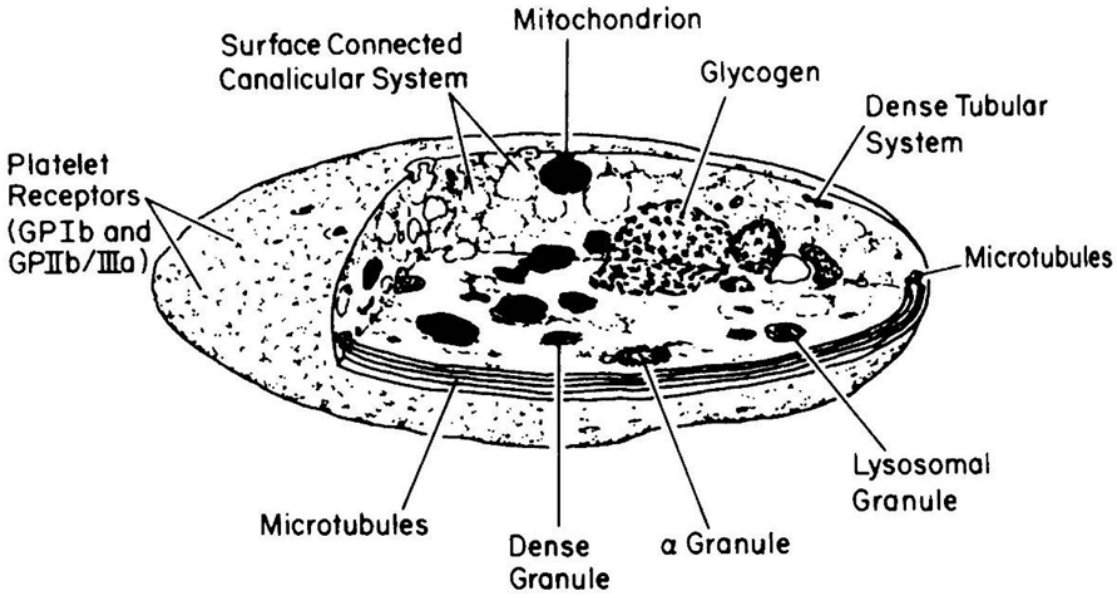


Figure 2.2. Structure of human platelet (Hanson 2004).

Secondary hemostasis is responsible for stabilizing the platelet aggregate by granular secretion and complex interaction between platelet membrane, enzymes, and coagulation factors. During secondary hemostasis, coagulation cascade activates the coagulation factors and leads to the conversion of fibrinogen to fibrin, which stabilizes the clot. Figure 2.3 is a schematic representation of platelet reactions when exposed to a stimulus.

2.2.1 Platelet Adhesion and Activation

The first step in blood coagulation is platelet adhesion. When blood is exposed to an injured vessel, platelets adhere to the surface by an interaction between the glycoprotein Ib (GP Ib) on the platelet surface and the connective tissue elements (especially collagen) that are exposed mediated by von Willebrand factor (vWF) as a required cofactor (Colman *et al.* 2005). When blood is exposed to a foreign surface, platelets adhesion may be mediated through glycoprotein IIb/IIIa as well as GP Ib-vWF interaction (Hanson 2004). GP IIb/IIIa is the platelet receptor for adhesive plasma proteins such as fibrinogen, vWF, fibronectin and vitronectin (Gresele *et al.* 2002). These plasma proteins aid in cell attachment. This binding usually occurs when GP IIb/IIIa has undergone a conformational change due to platelet activation. Platelets in the resting state do not bind these proteins. Platelets are activated when they come in contact with an injured blood vessel or a foreign surface or a platelet agonist such as ADP, 5-HT and thromboxane A_2 (TXA_2). Unactivated GP IIb/IIIa may also bind to surface proteins that have undergone conformational changes (Hanson 2004).

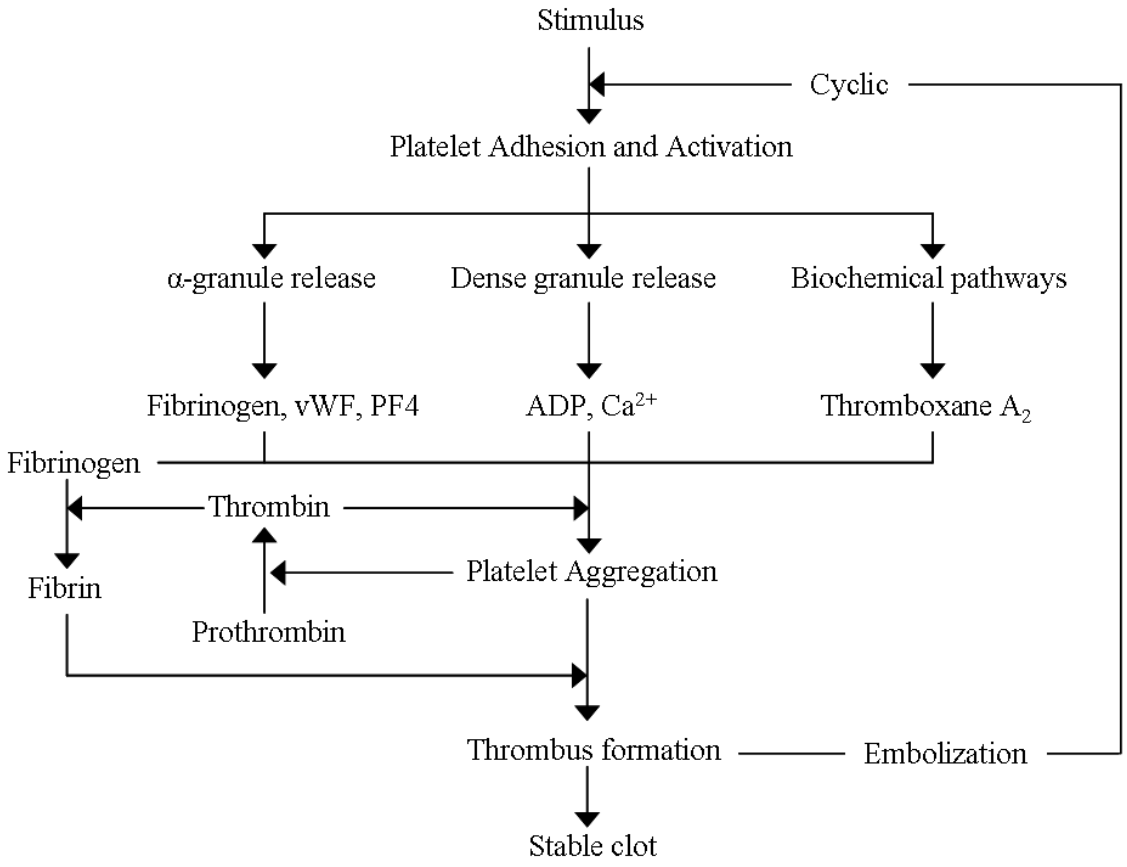


Figure 2.3. Platelet reactions to a stimulus (injured blood vessel or contact to foreign surface). Figure modified from (Adams 1985)

2.2.2 Platelet Aggregation

Platelet aggregation occurs within one minute of activation (Savi and Herbert 1996). After platelet adhesion, the following reactions occur: (1) the release of dense granule ADP, (2) formation of small quantities of thrombin and (3) activation of platelet biochemical processes leading to the generation of TXA_2. ADP, thrombin and thromboxanes, released into the blood plasma, act as agonists that activate more platelets. Due to this more, platelets are recruited to form a platelet aggregate. Platelet activation caused by these agonists results in the binding of activated GP IIb/IIIa on the platelet surface to the plasma proteins (primarily fibrinogen) that promote platelet aggregation. Platelet-platelet interactions develop when the GPIIb/IIIa complex is activated to bind fibrinogen. Each molecule of fibrinogen recognizes two GPIIb/IIIa complexes, which allows Ca^{2+}-dependant molecular bridges to form between two adjacent platelets (Perutelli and Mori 1992). Thrombin also binds to the platelet thrombin receptors. This results in (1) activating platelets, which then catalyze the formation of more thrombin, (2) stimulating ADP release and TXA_2 formation and (3) stimulating the formation of fibrin, which stabilizes the thrombus.

2.2.3 Granular Secretion

The release of substances stored in the platelet granules to the blood plasma is called granular secretion. Some of the physiologically important granules include ADP, collagen, epinephrine and thrombin. These granules act as release-inducing agents by

interacting with the platelets through specific receptors on the surface. α-granules such as platelet factor 4 (PF4), β-TG, vWF, fibronectin and fibrinogen are released by relatively weak agonists like ADP. Dense granules such as adenosine diphosphate (ADP), Ca^{2+} and serotonin (5-HT) are released by stronger agonists like thrombin. Agonist binding to platelets also initiates the formation of TXA_2, activation of contractile-secretory apparatus and mobilization of calcium from intracellular storage sites. This granule secretion and release of ADP promotes further aggregation through autocrine and paracrine stimulation and leads to blood coagulation (Holmsen 1994).

2.2.4 Platelet Coagulant Activity

Platelet aggregation also results in expression of negatively charged membrane phospholipids. This accelerates factor X activation and the conversion of prothrombin to thrombin. Hence, the platelet aggregate surface acts as a site for rapid synthesis of thrombin in excess of the neutralizing capacity of blood anticoagulant mechanisms. Thrombin is also required for polymerizing fibrinogen to form fibrin. Fibrin polymer then forms a stable clot.

2.2.5 Coagulation Cascade

Blood coagulation proceeds through a cascade of reactions that occur following a stimulus (injured vessel or contact to foreign surface). The coagulation factors (serine proteases) present in the inactive form in blood get activated due to the stimulus or

proteolytic cleavage by other coagulation factors. These active coagulation factors continue the series of reactions resulting in a cascade. Figure 2.4 shows a schematic of the blood coagulation cascade.

The coagulation cascade can either be intrinsic (when exposed to foreign surface) or extrinsic (injured vessel). The cascade mechanisms are well established (Ratnoff 1983; Hanson 2004). During intrinsic cascade, adhesion of contact factors to a negatively charged surface results in activation of factor XII (Hageman factor). The activated factor XII (XIIa) converts prekallikrein to kallikrein. More factor XIIa and kallikrein are produced by reciprocal activation. Factor XIIa also activates factor XI (Rosenthal's factor) to form XIa. Both prekallikrein and factor XI bind to a cofactor, high-molecular weight kininogen (HMWK). Factor IX (Christmas factor) is activated by factor XIa in a calcium-dependent step. Factor IXa activates factor X (Stuart-Prower factor) using factor VIIIa as a cofactor. Factor VIIIa is formed from factor VIII (antihaemophilic globulin) by the activity of an enzyme such as thrombin (Factor IIa). In the extrinsic cascade, factor VII (proconvertin, serum prothrombin conversion accelerator) is activated by tissue factor, a cell membrane protein that becomes available when underlying vascular structures are exposed to flowing blood due to injury. Factor VIIa acts as the extrinsic pathway activator of factor X. The common pathway begins when factor X is activated to form factor Xa. Factor Xa forms a complex with factor Va, a cofactor. Factor Va is formed from factor V (proaccelerin, ac-globulin, labile factor) by the activity of an enzyme such as thrombin. Factor Xa-Va complex converts prothrombin to thrombin, in the presence of calcium and platelet phospholipids. In addition to activating factors VIII and V, thrombin also activates factor XIII (fibrinase, fibrin stabilizing factor) and

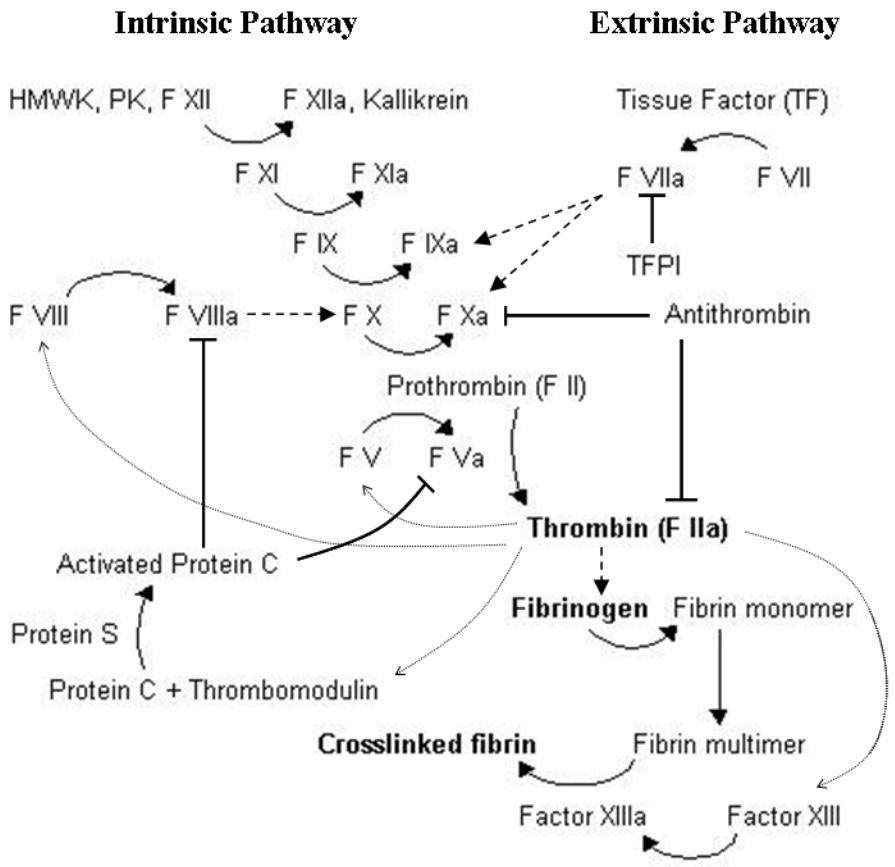


Figure 2.4. Blood coagulation cascade.⟶ represents conversion or activation of factors, –| represents inhibition, - - - - ► represents reactions catalyzed and ⋯⋯> represents the other functions of thrombin. Image adapted from Wikipedia. HMWK – high molecular weight kininogen, PK – prekallikrein, F – factor, TFPI – tissue factor pathway inhibitor. Figure from (Jfdwolff 2004)

catalyzes the polymerization of fibrinogen to fibrin. Factor XIIIa stabilizes the fibrin polymer via cross-linking to form a stable clot.

2.3 Anti-thrombotic control mechanisms

Multiple mechanisms exist in blood and vasculature to control the platelet adhesion and aggregation to healthy blood vessels. These mechanisms also control the size of the clot. These control mechanisms regulate blood coagulation in such a way that it is a highly localized and transient phenomenon. Once the injured blood vessels heal, the blood clot is removed by natural mechanisms. Understanding these control mechanisms would aid in developing materials that utilize these natural pathways to improve haemocompatibility.

At least five different types of control mechanisms need to be considered. First, blood flow may reduce the concentration of precursors and activated materials (Hanson 2004). This would reduce the likelihood of a blood clot growing in size. Second, many of the blood coagulation cascade reactions are catalyzed by surface contact (Hanson 2004). These reactions include the activation of factor X by factor VIIa and activation of prothrombin. Third, naturally occurring inhibitors for coagulation factors could stop the cascade and regulate blood coagulation (Hanson 2004). Antithrombin, for example, can inhibit thrombin and other coagulation enzymes. Another example would be tissue factor pathway inhibitor (TFPI), which inhibits factor VIIa. Fourth, during the coagulation cascade, enzymes are generated to degrade coagulation factors (Ratnoff 1983). Thrombomodulin, a transmembrane protein, forms a complex with thrombin and

activates protein C, which in turn inhibits cofactors V and VIII. Fibrinolytic enzyme plasmin activated during coagulation degrades fibrinogen, fibrin and also inactivates cofactors V and VIII. Fifth, platelet reactivity is controlled by the endothelium via biochemical systems (Marcus *et al.* 2005). The endothelium synthesizes fluid phase reactants intracellularly called autacoids, which are released when the cells come in contact with agonists. These autacoids lead to the formation of prostacyclin, nitric oxide (NO) and endothelial ecto-adenosine phosphatase (ADPase/CD39/NTPDase), which regulate blood coagulation (Marcus *et al.* 2003; Raggio and Morris 2004; Lwaleed and Bass 2005; Wise 2007).

The control mechanisms can also be classified into two broad categories, namely, late thromboregulators and early thromboregulators (Marcus *et al.* 2003). Late thromboregulators apply the effects after thrombin formation and early thromboregulators inhibit the events preceding thrombin formation. Antithrombin, tissue factor pathway inhibitor, protein C pathway and fibrinolysis are all classified as late thromboregulators (Marcus *et al.* 2003). Prostacyclin, nitric oxide and ecto-ADPase/CD39/NTPDase are classified as early thromboregulators (Marcus *et al.* 2003). For the purpose of developing a haemocompatible material, detailed understanding of naturally occurring inhibitors, enzymes that degrade coagulation factors and endothelium regulation is important.

2.3.1 Naturally occurring inhibitors

Antithrombin III (ATIII) was the first plasma inhibitory agent that was delineated (Ratnoff 1983). ATIII is the primary inhibitor of thrombin and factor Xa (Figure 2.4). ATIII also inhibits other activated coagulation factors to a lesser degree. ATIII forms a stoichiometric complex with the activated enzymes (Ratnoff 1983). The critical role of ATIII in regulating blood coagulation is evident from the frequency of thrombosis in people with a deficiency of this inhibitor (Ratnoff 1983). Heparin and heparan sulfate can augment the rate of ATIII inhibition of these enzymes by several thousand-fold (Raggio and Morris 2004). Heparin, a negatively charged sulfated polysaccharide, forms a complex with ATIII and inhibits thrombin. Without ATIII, heparin isn't a significant anticoagulant (Ratnoff 1983). Heparin is widely used for prevention and treatment of thrombotic states.

α_1-Antitrypsin is a glycoprotein synthesized in the liver. This protein acts as a protease inhibitor. *In vitro* studies show that α_1-antitrypsin inhibits factor XIa, thrombin, kallikrein and plasmin. But the biological significance of this protein in blood coagulation is not well established because deficiency of α_1-Antitrypsin doesn't cause any disturbance in clotting or fibrinolysis (Ratnoff 1983). However, deficiency of α_1-Antitrypsin causes obstructive pulmonary disease or cirrhosis of the liver (Wise 2007).

Tissue factor pathway inhibitor (TFPI) is a single-chain polypeptide which plays an important role in regulating tissue factor induced blood coagulation. TFPI is an endogenous anticoagulant protein, a serine protease inhibitor and is the only known regulator of the TF-dependent pathway of blood coagulation (Lwaleed and Bass 2005).

TFPI inhibits factor VIIa-TF complex in a factor Xa dependent manner (Figure 2.4) (Lwaleed and Bass 2005). Thus, TFPI prevents further activation of factor X in a feedback mechanism dependent on the concentration of factor Xa. In the presence of TFPI, additional factor Xa can be produced only via the intrinsic pathway. TFPI can inhibit activation of factor IX by factor VIIa-TF complex in the absence of factor Xa also. But this is achieved by a two-step process: TFPI binds to and inactivates factor Xa (Broze 1995) and this complex inhibits the factor VIIa-TF complex (Moncada *et al.* 1990; Gurewich 2000). TFPI does not inhibit the intrinsic pathway that is triggered by exposure to foreign surfaces. Hence, TFPI may an effective molecule for improving haemocompatibility.

2.3.2 Protein C Pathway

Thrombomodulin (TM), a transmembrane protein prevalent on the surface of endothelial cells, acts as a cofactor for thrombin induced activation of protein C. TM functions as a high affinity receptor for thrombin, which binds with a 1:1 stoichiometry. This thrombin-thrombomodulin complex activates protein C. Activated protein C degrades factors Va and VIIIa (Figure 2.4). *In vivo*, the protein C pathway is a vital anticoagulant pathway (Esmon 2003; Colman *et al.* 2005). In addition to activating protein C, thrombin bound to TM shows decreased ability to convert fibrinogen to fibrin and stimulate platelets (Esmon 1989). The binding of thrombin to TM also accelerates inhibition of thrombin by ATIII (Preissner *et al.* 1987).

2.3.3 Fibrinolysis

Fibrinolysis is carried out by the production of the enzyme plasmin from the inactive proenzyme plasminogen. Plasminogen is activated by two-chain tissue plasminogen activator (t-PA) or two-chain urokinase plasminogen activator (u-PA). These activators are secreted as single chain molecules that are converted to two-chain activators by plasmin (Cesarman-Maus and Hajjar 2005). The generated plasmin degrades the fibrin clot and extracellular matrix molecules (Gurewich 2000). Both t-PA and u-PA can be inhibited by plasminogen activator inhibitors (PAI) (Cesarman-Maus and Hajjar 2005), while plasmin is inhibited by its major inhibitor, α_2-antiplasmin, and to a lesser extent by α_2-macroglobulin (α_2-MG) (Cesarman-Maus and Hajjar 2005). Plasmin is then rapidly inhibited unless it remains bound to fibrin or to its cell surface receptors. Figure 2.5 shows a simplified fibrinolytic pathway.

2.3.4 Prostacyclin

Prostacyclin (PGI_2) is an early thromboregulator (Marcus *et al.* 2003) and the most potent endogenous inhibitor of platelet aggregation (Moncada *et al.* 1990). PGI_2 is the main product formed in endothelial cells during arachidonic acid metabolism by the action of cyclooxygenase system with PGI_2 synthase (Lim and Dey 2002). First, arachidonic acid is released from the phospholipid pools by phospholipase A_2. This arachidonic acid is converted to prostaglandin H_2 by the action of cyclooxygenase. Prostaglandin H_2 is then converted to PGI_2 by PGI_2 synthase (Vane and Botting 1995).

Endothelial cells are the major source of PGI_2 in the vasculature. PGI_2 helps in regulating platelet aggregation and vasodilation (Jin *et al.* 2005). PGI_2 release can be stimulated by mechanical and chemical stimuli. PGI_2 has a half life of 60 minutes in plasma and it loses the inhibitory effects on platelet aggregation in about 30 minutes (Moncada 1982; Jin *et al.* 2005). PGI_2 inhibits platelet aggregation via a cAMP-Protein Kinase A signal transduction mechanism. PGI_2 interacts with a specific G protein receptor on the platelet surface, resulting in the activation of adenylate cyclase (Jin *et al.* 2005). Activation of adenylate cyclase increases the production of cyclic adenosine monophosphate (cAMP) in platelets (Gorma *et al.* 1977; Tateson *et al.* 1977). Increase in cAMP concentrations causes phosphorylation of vasodilator-stimulated phospho-protein (VASP) via protein kinase A and inhibition of Ca^{2+} mobilization and granule release (Aszódi *et al.* 1999).

VASP regulates fibrinogen receptor inactivation resulting in inhibition of fibrinogen binding and fibrinogen-dependent platelet cross-linking (Horstrup *et al.* 1994). Inhibition of Ca^{2+} mobilization and granule release prevents further platelet activation and aggregation (Jin *et al.* 2005). In addition to inhibiting platelet aggregation, PGI_2 also returns the activated platelets to a rested state (Jin *et al.* 2005). Usually PGI_2 is used to inhibit platelet activation and aggregation during preparation of platelet suspensions (Shahbazi *et al.* 1994; Radomski *et al.* 1996). Modifying the blood-contacting surface of the biomaterial by attaching prostacyclin might not be efficient because of the low half-life. Also, prostacyclin doesn't play an important role in inhibiting platelet adhesion (Higgs *et al.* 1978).

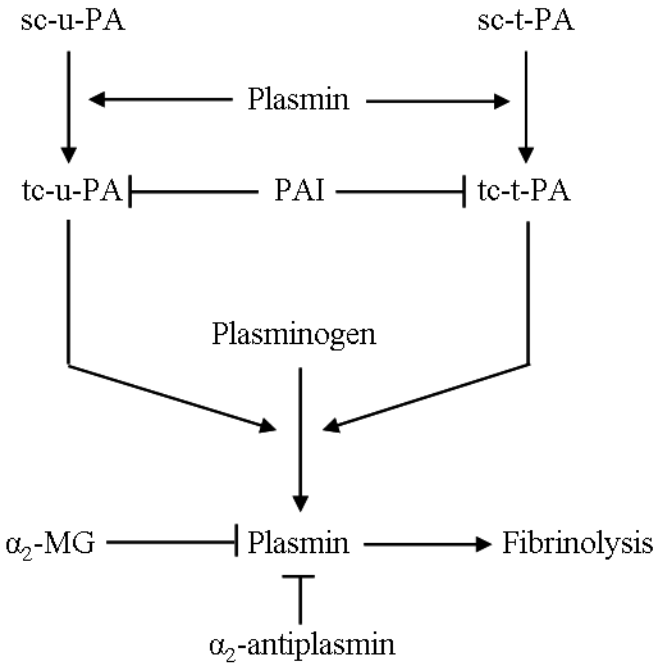


Figure 2.5. A simplified schematic of fibrinolysis. –| represents inhibition and → represents reactions. sc-u-PA – single chain urokinase plasminogen activator, sc-t-PA – single chain tissue plasminogen activator, tc-u-PA – two chain urokinase plasminogen activator, tc-t-PA – two chain tissue plasminogen activator, PAI – plasminogen activator inhibitor, α_2-MG – α_2-macroglobulin. Figure adapted from (Cesarman-Maus and Hajjar 2005)

2.3.5 Nitric Oxide (NO)

In 1980, Furchgott and Zawadzki discovered the presence of an endothelium-derived relaxing factor (Furchgott and Zawadzki 1980), which was later identified to be nitric oxide (Palmer *et al.* 1987; Ignarro *et al.* 1999). Nitric oxide has been identified to play a vital role in regulating vasodilation, vascular permeability, neurotransmission and antithrombotic processes in the vasculature (Palmer *et al.* 1987). Nitric oxide is synthesized in different cells including endothelium, macrophages and platelets (Giustarini *et al.* 2009). Nitric oxide is synthesized enzymatically during the conversion of L-arginine to L-citrulline by the action of a class of enzymes called nitric oxide synthases (NOS). NOS requires flavin mononucleotide, flavin adenine dinucleotide and tetrahydrobiopterin as cofactors and nicotinamide adenine dinucleotide phosphate and molecular oxygen as co-substrates (Marletta 1994). NOS is classified into three types based on the location and pattern of expression and dependence of Ca^{2+}: nNOS (NOS I) – neuronal NOS expressed primarily in the neurons, iNOS (NOS II) – inducible NOS expressed in macrophages, smooth muscle cells and neutrophils and eNOS (NOS III) – endothelial NOS expressed primarily in the endothelial cells. Activities of nNOS and eNOS depend on Ca^{2+}. iNOS is less dependent to the Ca^{2+} concentrations. NO synthesis by iNOS is regulated at the transcription levels.

Platelets also synthesize and release NO. The levels of NO release is increased multiple fold by platelet activation. It has been shown that platelets in the rested state release up to 20 nM NO and activated platelets release 5-15 µM NO (Freedman *et al.* 1997). This NO released in activated platelets inhibit platelet recruitment for further

aggregation (Freedman *et al.* 1997). This platelet-derived NO could play a major role in regulating blood coagulation.

NO acts in multiple ways to control blood coagulation. NO shows high affinity towards heme iron and binds to heme containing proteins including soluble guanylate cyclase (sGC). NO bound sGC catalyzes the conversion of guanosine triphosphate (GTP) to 3, 5-cyclic guanosine monophosphate (cGMP). cGMP activates cGMP dependent kinase that phosphorylates specific proteins leading to platelet inhibition (Mellion *et al.* 1981). NO has also been associated with decreasing the intracellular levels of Ca^{2+} (Jin *et al.* 2005). Lower Ca^{2+} concentration results in inhibition of cytoskeletal rearrangement, α- and dense granule secretion and overall platelet activation (Jin *et al.* 2005). cGMP, produced by the action of NO, and cGMP-dependent protein kinase inhibit the TXA_2 mediated platelet activation by catalyzing the phosphorylation of TXA_2 receptors (Wang *et al.* 1998). Studies have suggested that NO attenuates the expression of P-selectin, a protein required for platelet adherence to the endothelium, via cGMP-dependent protein kinase (Davenpek *et al.* 1994; Maurohara *et al.* 1995). NO can also slow down the cross-linking of activated platelets by reducing the number of GPIIb/IIIa receptors on the platelet surface (Mendelsohn *et al.* 1990) and by increasing the dissociation constant of the receptor for fibrinogen (Jin *et al.* 2005). Due to these effects, platelet-platelet interaction is reduced as the activated platelets can't bind with the fibrinogen, which is required for forming molecular bridges. NO also diffuses readily to act on the other surrounding platelets. Figure 2.6 is a schematic representation of the multiple roles of NO in platelet inhibition.

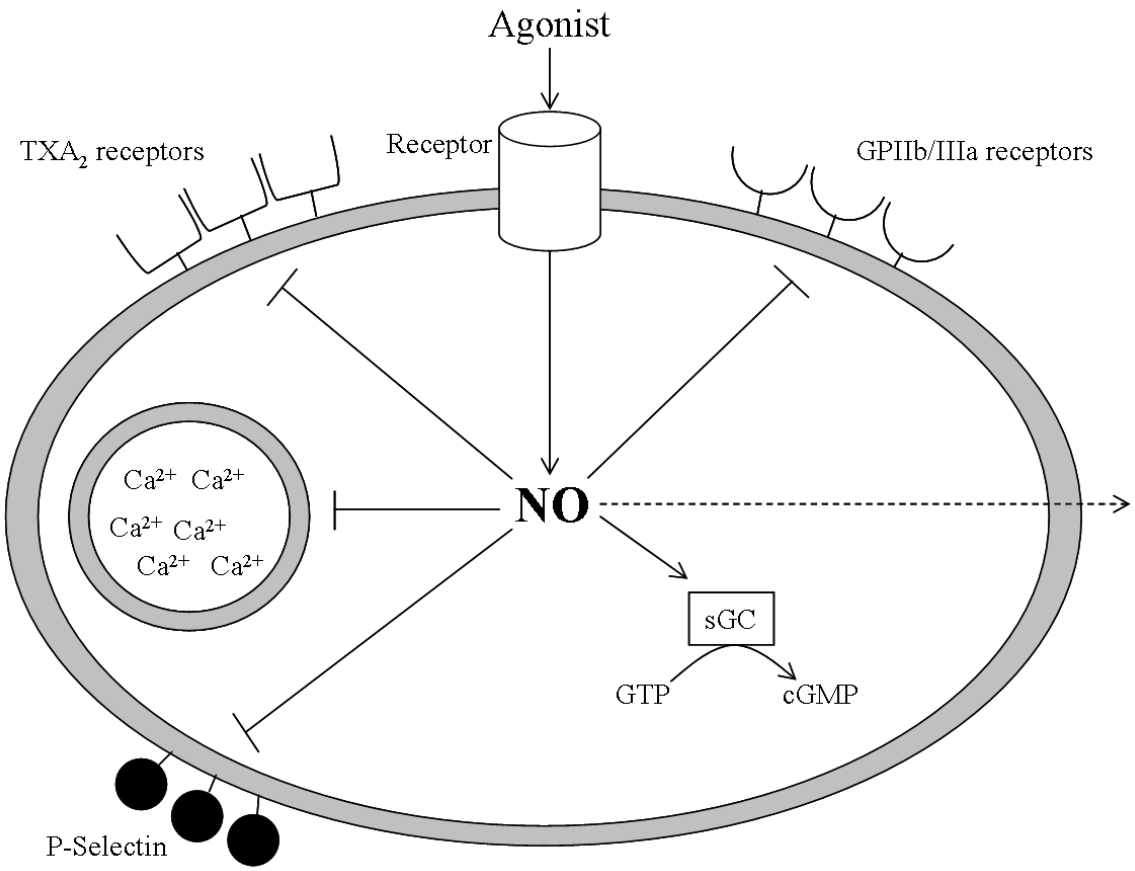


Figure 2.6. Effects of nitric oxide on platelet inhibition. NO – nitric oxide, TXA_2 receptors – thromboxane A_2 receptors, sGC – soluble guanylate cyclase, GTP – guanosine triphosphate, cGMP – cyclic guanosine monophosphate. → represents activation or formation, –| represents inhibition or suppression and -⇢ represents diffusion. Figure adapted from (Jin *et al.* 2005)

2.3.6 Endothelial ecto-adenosine phosphatase (ADPase/CD39/NTPDase)

Platelets in the circulating blood are maintained at the rested state by the action of prostacyclin and NO released by endothelial cells. As discussed in blood coagulation, when the platelets are exposed to a stimulus, platelets adhere to the surface, get activated and release ADP and other agonists such as TXA_2 and 5-HT. ADP acts as a paracrine signal for recruiting other platelets and forming a thrombus (Dai *et al.* 2004). This ADP-induced platelet aggregation is regulated by the action of a nucleotidase, CD39 (Gayle III *et al.* 1998; Weksler 2000).

Although the presence of a nucleotidase on the plasma membrane of cells has been known for over 50 years (Rothstein *et al.* 1953), the identification of these enzymes was done in the last decade (Plesner 1995; Handa and Guidotti 1996). ADPase/CD39/NTPDase is membrane glycoprotein initially found in the lymphoid cells (Jin *et al.* 2005). CD39 hydrolyzes nucleotide tri and/or diphosphates in the presence of bivalent cations such as Ca^{2+} and Mg^{2+} (Kaczmarek *et al.* 1996; Wang and Guidotti 1996). CD39 is an integral membrane protein with two transmembrane domains – a small amino and carboxy terminal in the cytoplasm and a large extracellular domain that is responsible for the enzymatic activity (Treuheit *et al.* 1992; Stout and Kirley 1994). Micromolar concentrations of ADP, released during dense granule secretion by the platelets, cause the platelets to change shape from smooth discoids to spiculated spheres, leading to platelet aggregation (Savi and Herbert 1996; Dai *et al.* 2004). ADP binds to the P_2 purine receptors on platelets, causing platelet aggregation (Savi and Herbert 1998).

CD39 rapidly metabolizes ADP released from activated platelets resulting in return of platelets to the resting state (Marcus *et al.* 1997). ADP metabolized by CD39 forms adenosine monophosphate (AMP), which is further metabolized to form adenosine by the action of 5'-nucleotidase (Zimmerman 1992). Adenosine activates adenylate cyclase and triggers the cAMP-dependent inhibition of platelet aggregation (Marcus and Safier 1993). These actions of CD39 modulates intravascular platelet accumulation and helps localize thrombus formation to the setting of tissue injury (Marcus and Safier 1993). Some studies have demonstrated that CD39 also blocks the binding of fibrinogen or von Willebrand factor to platelets by inhibiting the activation of platelet glycoprotein IIb/IIIa adhesion receptors (Makita *et al.* 1998). CD39 maintains vascular fluidity in the complete absence of PGI_2 and NO, indicating that the latter are ancillary components of thrombosis (Marcus *et al.* 2005). Studies done on three different animal models have shown that CD39 is an effective thromboregulator purely by acting on the platelet releasates (Marcus *et al.* 2003; Marcus *et al.* 2005). Figure 2.7 shows the mechanism by which CD39 inhibits platelet aggregation. Metabolic removal of ADP secreted by the activated platelets has potential to be used as an effective anti-thrombotic mechanism for enhancing haemocompatibility.

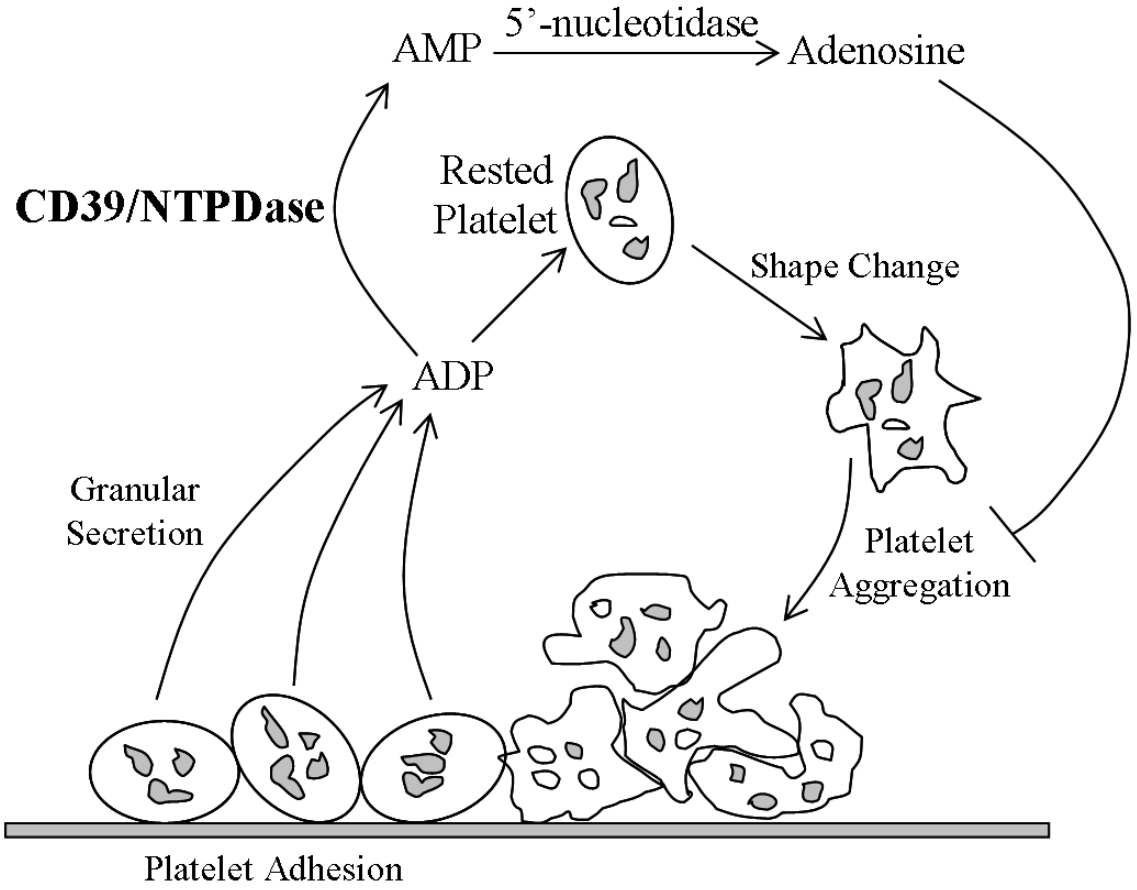


Figure 2.7. Mechanism of platelet inhibition by CD39/NTPDase by regulating ADP-induced platelet aggregation. → represents formation or release and –| represents inhibition.

Enzymes that catalyze hydrolysis of ADP to form AMP have also been identified in the salivary glands of arthropods (Basanova *et al.* 2002), which inhibit ADP-induced blood coagulation to facilitate blood feeding, the endoplasmic reticulum of rats, which plays an intracellular role in glycosylation (Failer *et al.* 2002), skeletal muscle transverse tubules in rabbits (Treuheit *et al.* 1992), gizzard smooth muscles in chicken (Stout and Kirley 1994), vascular endothelium, smooth muscle, spleen and lungs of pigs (Lemmens *et al.* 2000), stems of garden pea (*Pisum sativum*) (Shibata *et al.* 1999) and potato tubers (*Solanum tuberosum*) (Handa and Guidotti 1996). Similar nucleotidases have also been identified and characterized in a number of other organisms including *Caenorhabditis elegans* (Ucceletti *et al.* 2008), baker's yeast (*Saccharomyces cerevisiae*) (Gao *et al.* 1999), fission yeast (*Schizosaccharomyces pombe*) (D'Alessio *et al.* 2003) and *Toxoplasma gondii* (Asai *et al.* 1995). Immobilizing one of these enzymes to the surface of a biomaterial would potentially improve the haemocompatibility by inhibiting ADP-induced platelet aggregation.

2.4 Current technologies used to improve haemocompatibility

Currently many techniques are employed to improve haemocompatibility of biomaterials. These methods can be classified into three broad techniques, namely, (1) increasing surface hydrophilicity, (2) disguising material surfaces and (3) attaching biologically active molecules. Surface hydrophilicity can be enhanced by grafting long hydrophilic chains (Golander and Kiss 1988; Brinkman *et al.* 1989; Nojiri *et al.* 1990) or incorporating hydrogels to the surface. The main problem with this approach is that when

the surface interacts with the surroundings, these long chains create high steric hindrance that affects the biological properties of the biomaterials. The retention of large quantities of water by hydrogels makes the surface similar to biological tissues. However, low cell adhesion, because of hydrogels, does not translate to low thrombogenicity as a number of hydrogels used as dialysis membrane materials are recognized as thrombogenic (Sefton 1990). Surfaces that come in contact with blood can also be disguised so that they are not recognized as a foreign material by blood. Some of the common procedures are endothelialization (Herring *et al.* 1984), albumin passivation (Kottke-Marchant *et al.* 1989; Amiji *et al.* 1992), and phopholipid mimicking surfaces (Durrani *et al.* 1986; Ishihara *et al.* 1991; Iwasaki *et al.* 1996; Ishihara *et al.* 1999a; Ishihara *et al.* 1999b). But, preparing surface disguised biomaterials in bulk and storing them could be a challenge. Also, this technique doesn't stop platelet activation when the material is used in extracorporeal systems (Borgdorff *et al.* 1999).

Attaching biologically active molecules to improve haemocompatibility is a commonly used procedure. Some commonly used compounds are heparin (Heyman *et al.* 1985; Han *et al.* 1989), hirudin (Seifert *et al.* 1997), nitric oxide (Tullett and Rees 1999) and NO-releasing compounds (Smith *et al.* 1996). Coating surfaces with heparin (Gott *et al.* 1963) is one of the most popular techniques in improving haemocompatibility. In addition to the effect on ATIII, heparin also helps in the formation of a blood-friendly secondary superficial membrane (Vroman 1988; Wendel and Ziemer 1999). Heparin has been attached to different surfaces physically, ionically and covalently. Ionic binding of heparin has been used in the commercial manufacture of polyurethane catheters (Angiocath, Toray Industries) and cardiopulmonary bypass circuits (Baxter Bentley

Healtcare Systems). However, if heparin is bound ionically to a surface, it can be slowly released over time (Magnani and Piras 2008). Covalent binding of heparin leads to loss of activity. Surface coating with heparin shows improved haemocompatibility over a period of time. However, uncontrolled release, loss of heparin activity over time and consumption of plasma coagulation factors limit its use (Larsson *et al.* 1987). Direct inhibition of thrombin can be achieved by immobilizing molecules such as hirudin (Seifert *et al.* 1997). Although hirudin improves haemocompatibility and doesn't bind with plasma coagulation factors, the long term use is very limited due to the short half life of 1 – 1.5 h (Nowak *et al.* 1992).

Protein C pathway has been utilized to improve haemocompatibility by covalently immobilizing thrombomodulin (Sperling *et al.* 2004). By attaching thrombomodulin to the surface of poly(ethylene glycol), Sperling et al. proved improved haemocompatibility of the material. But this method could pose a serious limitation. Activated protein C, which has a half-life of 20 – 30 minutes (Sperling *et al.* 2004), may be transported by blood flow and it could inhibit clot formation on an injured vessel. This could cause hemorrhage. In short, this procedure might not have the desired site-specificity.

Fibrinolytic pathway can be used to enhance haemocompatibility of materials. Attachment of plasmin to the material would not provide sustained haemocompatibility as plasmin has a very short half life (Wiman and Collen 1978). However, the fibrinolytic pathway may be used to enhance haemocompatibility by attaching plasminogen activation factors to the surface. Antithrombotic mechanisms of a polymer surface with immobilized urokinase have been studied (Sugitachi and Takagi 1978; Ohshiro and Kosaki 1980; Kitamoto *et al.* 1987; Kitamoto *et al.* 1991). The results showed enhanced

haemocompatibility of the polymers. However, urokinase is not an easily available protein and hence, using it for mass production of blood-contact materials might not be economically feasible. Also, action of urokinase takes effect only in the fibrinolysis process. Due to this, the formation of blood clots is not prevented, but the clots formed are degraded by the action of plasmin activated by the urokinase-modified material.

An earlier study has applied the concept of NO inhibition of platelet adhesion to improve haemocompatibility by incorporating NO-releasing compounds in polymers which release NO following contact with blood (Saavedra *et al.* 1996). Another study demonstrated the covalent attachment of NO-releasing compounds to polymeric matrices in which the time-course of NO release was variable (Smith *et al.* 1996). The major drawback of these techniques is the exhaustion of NO molecule. This limitation can be overcome by utilizing endogenous NO. Although the free NO in blood stream is only 3 nM, the concentration of S-nitrosothiols (RSNO) is 7 μM and is primarily composed of S-nitrosoalbumin (Stamler *et al.* 1992). These S-nitrosothiols are considered to be endogenous reservoirs of NO. It is established that in solution the NO associated with S-nitroso albumin preferentially transferred to cysteine (Meyer *et al.* 1994). Earlier work done in this lab has shown that attaching cysteine to PET and polyurethane inhibits platelet deposition by 67% (Duan and Lewis 2002). These results show that the haemocompatibility is enhanced by modifying the surface of the biomaterial with cysteine.

Bakker et al. used metabolic removal of ADP to improve haemocompatibility of polymer surfaces by coating polyurethane vascular prostheses with NTPDase from potato (Bakker *et al.* 1991; van der Lei *et al.* 1992). When these NTPDase coated prostheses

were implanted to the peritoneal cavity of rats (Bakker *et al.* 1991) and carotid arteries in rabbits (Bakker *et al.* 1991; van der Lei *et al.* 1992), results show that NTPDase coating on prostheses improves haemocompatibility of biomaterials. However, their results showed that one of the prostheses, coated with NTPDase, was completely occluded when the implant was taken out after 3 weeks (van der Lei *et al.* 1992). This shows that the NTPDase coated polymer lacked prolonged haemocompatibility due to the release of NTPDase into the blood stream. Another study has shown that covalent attachment of NTPDase to biopolymer surfaces enhance platelet inhibition (Marconi *et al.* 1983). When NTPDase immobilized rings were placed in a dog's femoral vein for 15 days, the materials showed significant haemocompatibility (Marconi *et al.* 1983). The activity of NTPDase immobilized on the biopolymer was approximately 0.7 μM of inorganic phosphate released in an hour per cm^2 (Marconi *et al.* 1983). However, the surface modification techniques used by Marconi et al. were shown by various studies to cause surface deterioration and weakening of mechanical properties (Ellison *et al.* 1982; Sanders and Zeronian 1982; Kao *et al.* 1998; Liu *et al.* 2005). These changes in surface and bulk properties could limit the applications of the haemocompatible material. Based on these studies, it is expected that covalently attaching NTPDase to biopolymer surfaces, using a surface modification technique that would not significantly alter the surface and bulk properties of the material, would be an effective solution for minimizing the current limitations and enhancing platelet inhibition by metabolizing ADP (Marcus *et al.* 1997). This technique would provide effective site-specific platelet inhibition and potentially enhance haemocompatibility.

The combined effects of NO and NTPDase on platelet deposition has been studied in solution (Ramamurthi *et al.* 2001). Ramamurthi et al. performed an in vitro study to compare the inhibition of platelet deposition to a biomaterial by NO in the absence and presence of soluble NTPDase. The results of these platelet inhibition studies with NO and NTPDase show that the inhibitory effects of NO and NTPDase are additive (Ramamurthi *et al.* 2001). The platelet inhibitory effect of NO was enhanced by the presence of NTPDase in a dose-dependent manner (Ramamurthi *et al.* 2001). Effective immobilization of NTPDase and cysteine to the surface of a biomaterial would block ADP-induced platelet aggregation and promote NO mediated platelet inhibition. This additive nature can be used to further enhance the haemocompatibility in biomaterials.

2.5 Polyethylene terephthalate – a model polymer

To study the effect of NTPDase attachment in improving haemocompatibility, NTPDase had to be attached successfully to a model polymer. Polyethylene terephthalate (PET) was chosen as the model polymer based on its wide range of biomedical applications in blood-contacting systems and desired mechanical strength and durability. PET is a linear, aromatic polyester first manufactured by Dupont in 1940s. PET is used in vascular prostheses (Vinard *et al.* 1988; Kottke-Marchant *et al.* 1989; Haulon *et al.* 2003; Blanchemain *et al.* 2007a), heart valve sewing cuffs (Tweden *et al.* 1997; Illingworth *et al.* 1998; Jin *et al.* 2007), implantable sutures (Homsy *et al.* 1968; 21CFR878.5000 2008), surgical mesh (Zieren *et al.* 2004; Bracco *et al.* 2005) and components of percutaneous access devices (von Recum 1984). PET was first used in biomedical

applications as sutures in the 1950s (Taylor 1975). Clinical use of vascular prostheses made of woven and knitted PET has been prevalent since the 1960s (Lindenauer *et al.* 1976). The biomaterial properties of PET that make the polymer suitable for biomedical applications are biostability (Greenwald *et al.* 1994), promotion of tissue ingrowth (Feldman *et al.* 1983), a well understood fibrotic response (Tilney and Boor 1975; Müller and Dasbach 1994) and a long history of human implantation (Brun *et al.* 1992). PET is one of very few materials that has a long history of biomedical applications and is still being used in a wide range of applications from sutures to vascular prostheses.

For improving haemocompatibility of PET, NTPDase has to be covalently immobilized to the surface. However, PET is an inert polymer. Surface functional groups have to be introduced to attach NTPDase. Currently, many techniques such as hydrolysis (Ellison *et al.* 1982; Kao *et al.* 1998; Bide *et al.* 2006), reduction (Bùi *et al.* 1993), glycolysis (Fadeev and McCarthy 1998), aminolysis (Ellison *et al.* 1982; Avny and Rebenfeld 1986; Duan and Lewis 2002; Gappa-Fahlenkamp and Lewis 2005; Bide *et al.* 2006; Liu *et al.* 2008) and amination (Nissen *et al.* 2008) are used to introduce reactive functional groups on PET surfaces. Although these techniques effectively introduce reactive groups, surface modifications accompany the limitation of modifying surface and bulk properties of PET. Loss of mechanical strength is a common problem associated with surface modification due to degradation of the polymer (Ellison *et al.* 1982; Sanders and Zeronian 1982; Fukatsu 1992; Kao *et al.* 1998). This results in reducing the durability of PET in implants and other long term biomedical applications. Even with these limitations, many studies have demonstrated immobilization of active biomolecules (Kottke-Marchant *et al.* 1989; Bùi *et al.* 1993; Duan and Lewis 2002; Aggarwal *et al.*

2005; Liu *et al.* 2005; Phaneuf *et al.* 2005; Blanchemain *et al.* 2007a; Blanchemain *et al.* 2007b; Liu *et al.* 2008). These studies have also showed improved haemocompatibility (Heyman *et al.* 1985; Han *et al.* 1989; Kottke-Marchant *et al.* 1989; Duan and Lewis 2002; Liu *et al.* 2008), infection resistance (Illingworth *et al.* 1998; Aggarwal *et al.* 2005; Phaneuf *et al.* 2005; Blanchemain *et al.* 2007a; Blanchemain *et al.* 2007b), and cell adhesion (Liu *et al.* 2005). In addition to techniques such as aminolysis and hydrolysis, carboxylation has been performed to introduce reactive carboxyl groups to PET surfaces (Yang *et al.* 2000). However, the surface and bulk properties of carboxylated PET haven't been explored. Covalent immobilization of NTPDase to a functionalized PET surface with desired surface and bulk properties would fabricate a viable biomaterial with enhanced haemocompatibility.

CHAPTER 3

ANALYSIS OF FUNCTIONALIZED POLYETHYLENE TEREPHTHALATE WITH IMMOBILIZED NTPDase

Biomaterials developed for specific functions need to possess particular surface and bulk properties. When designing a biomaterial for blood-contact systems, it is critical to understand the surface properties of the material. Surface properties of the material could determine many biological reactions and responses, such as protein adsorption and blood compatibility. Surface properties also influence cell adhesion and cell growth, which would be important for the material to integrate with the body. The importance of surface properties on biological responses has been appreciated for a long time. In addition to surface properties, desired bulk properties such as mechanical strength, permeability and durability also influence the applications of biomaterials. Classic techniques have been developed by engineers and material scientists for studying the surface and bulk properties of biomaterials. This chapter discusses three different techniques to introduce reactive functional groups to polyethylene terephthalate and the associated effects of functionalization on the surface and bulk properties. Studies performed on immobilizing NTPDase on functionalized PET and the effectiveness of the immobilization are also discussed. Based on these studies, a preferred method of surface modification is identified and used for further exploration.

3.1 Introduction

For this research, it was important to choose a model polymer that had various clinical and biomedical applications. The polymer also had to be mechanically strong with desirable surface properties. Based on these requirements, polyethylene terephthalate (PET), a linear, aromatic polyester, was chosen. Many studies have shown that PET is a strong and durable polymer for biomedical applications (Bide *et al.* 2006; Nissen *et al.* 2008). PET also has a wide range of medical applications which include vascular prostheses (Vinard *et al.* 1988; Kottke-Marchant *et al.* 1989; Haulon *et al.* 2003; Blanchemain *et al.* 2007a), heart valve sewing cuffs (Tweden *et al.* 1997; Illingworth *et al.* 1998; Jin *et al.* 2007), implantable sutures (Homsy *et al.* 1968; 21CFR878.5000 2008) and surgical mesh (Zieren *et al.* 2004; Bracco *et al.* 2005). With several of the PET applications involving contact with blood, haemocompatibility is a major concern. An effective approach to improve haemocompatibility is to modify the surface with biologically active compounds that would inhibit platelet adhesion and aggregation. Such biologically active molecules can be immobilized on the polymer surfaces by physical adsorption or covalent binding. Physical adsorption would only provide a short-term effect as the material would lose its haemocompatibility once the biologically active molecule is lost into blood. Covalent binding provides the strongest immobilization. Hence, covalently binding a biologically active molecule would potentially improve and provide prolonged haemocompatibility. However, PET is an inert polymer and lacks active functional groups on the surface to covalently attach biologically active molecules. Hence, introducing reactive functional groups to the surface of PET is necessary for

immobilizing biomolecules that could potentially improve the haemocompatibility. Currently, many techniques such as amination (Nissen *et al.* 2008), reduction (Bùi *et al.* 1993), glycolysis (Fadeev and McCarthy 1998), hydrolysis (Ellison *et al.* 1982; Kao *et al.* 1998; Bide *et al.* 2006) and aminolysis (Ellison *et al.* 1982; Avny and Rebenfeld 1986; Duan and Lewis 2002; Gappa-Fahlenkamp and Lewis 2005; Bide *et al.* 2006; Liu *et al.* 2008) are used to introduce reactive functional groups on PET surfaces.

Although these surface functionalization techniques effectively introduce reactive groups, these processes alter the surface properties and the mechanical strength of PET. During surface functionalization, PET is degraded by the effect of chemical reactions and this causes significant loss of mechanical strength (Fukatsu 1992; Bùi *et al.* 1993; Kao *et al.* 1998; Liu *et al.* 2005; Nissen *et al.* 2008). These changes in the surface and bulk properties make the polymer less viable for biomedical applications, as the material no longer has the desirable mechanical strength required for long-term biomedical applications. However, many studies have shown that these surface functionalization techniques can be used to immobilize active biomolecules (Kottke-Marchant *et al.* 1989; Bùi *et al.* 1993; Duan and Lewis 2002; Aggarwal *et al.* 2005; Liu *et al.* 2005; Phaneuf *et al.* 2005; Blanchemain *et al.* 2007a; Blanchemain *et al.* 2007b; Liu *et al.* 2008). There are studies that have demonstrated improved haemocompatibility (Kottke-Marchant *et al.* 1989; Duan and Lewis 2002; Liu *et al.* 2008), infection resistance (Aggarwal *et al.* 2005; Phaneuf *et al.* 2005; Blanchemain *et al.* 2007a; Blanchemain *et al.* 2007b) and cell adhesion (Liu *et al.* 2005). For this reason, functionalizing PET surfaces with minimal changes to the surface and bulk properties would provide a valuable tool to improve the biological property of PET in biomedical applications. In the recent past, carboxylation

has been used to introduce reactive carboxyl groups to PET surfaces (Yang *et al.* 2000). However, the effect of carboxylation on the surface and bulk properties hasn't been studied. Understanding the effects of carboxylation on surface and bulk properties could potentially provide an effective PET surface modification.

When biomaterials are developed for medical devices and artificial organs, the surface and bulk properties of the material has to be studied. In blood contact systems, it is obvious that the surface properties significantly impact the haemocompatibility of the material, as only the surface comes in contact with blood. Although all the parameters that influence the biological response of surfaces are not completely understood, many studies have shown the importance of roughness, wettability, surface charge, etc (Ratner 2004). Many techniques have been developed for studying these surface properties. This study has analyzed carboxylated PET to understand the effects of the surface modification on surface and bulk properties, namely possible surface deterioration, surface roughness and mechanical strength. Also, two other commonly used techniques to introduce functional groups to PET surface, namely, aminolysis and hydrolysis were analyzed for comparison. During surface analysis, multiple pieces of information are required for constructing a picture of the surface. Hence, it is usually advised to use more than one technique to analyze surfaces. If the data obtained from these methods agree with one another, the results obtained would be reliable. Hence, in this work a scanning electron microscope (SEM) and atomic force microscope (AFM) have been used to study the surfaces, both qualitatively and quantitatively. High resolution images obtained from the SEM were used to understand the possible surface deterioration due to surface

functionalization. The surfaces were also analyzed using AFM to quantitatively estimate the surface roughness.

Bulk properties such as mechanical strength, permeability and elasticity play an important role in understanding the durability of a biomedical device or an implant. Mechanical strength is a critical parameter in determining the functionality and efficacy of the material in biomedical applications. The fundamental test for understanding mechanical strength is tensile testing. Mechanical strengths of aminolyzed, hydrolyzed and carboxylated PET were studied using tensile testing. The results were compared to the mechanical strength of unmodified PET.

As mentioned earlier, PET is used in many blood contacting systems. In such an environment, contact with a foreign material such as PET activates platelets and leads to platelet aggregation. Covalent or ionic binding of anticoagulant or other biologically active molecules to the reactive functional groups on modified PET can potentially improve the haemocompatibility. Studies have shown that nucleoside triphosphate diphosphohydrolase (NTPDase) improves haemocompatibility of polymers. NTPDase has been used to improve the haemocompatibility of polymers either by coating (Bakker *et al.* 1991; van der Lei *et al.* 1992) or by covalent binding (Marconi *et al.* 1983). When blood comes in contact with a foreign material, irreversible platelet aggregation is effected by the release of adenosine diphosphate (ADP) molecules in a concentration dependent manner (Meyers *et al.* 1979; Holmsen 1994; Riddell *et al.* 1997). NTPDase is an enzyme that has been shown to inhibit ADP-induced platelet aggregation by hydrolyzing ADP to AMP and inorganic phosphate (Pi) (Mans *et al.* 2000; Costa *et al.* 2004; Glenn *et al.* 2008).

Although NTPDase coating and covalent binding improved the haemocompatibility of the materials, both protocols had their limitations. In the case of coating polyurethane with NTPDase, a rapid release of the non-specifically bound NTPDase could result in undesired effects. Also, once the coated NTPDase is washed off, the polymer would lose its haemocompatibility. Earlier studies immobilized NTPDase to hydrolyzed PET (Marconi *et al.* 1983) despite hydrolyzed PET showing a loss of mechanical strength due to hydrolysis (Ellison *et al.* 1982; Sanders and Zeronian 1982; Kao *et al.* 1998; Liu *et al.* 2005). However, *in vivo* testing by inserting the NTPDase-modified hydrolyzed PET in a dog's femoral vein for 15 days showed that the polymer was haemocompatible (Marconi *et al.* 1983). Based on these studies, it is evident that immobilization of NTPDase to mechanically stable PET would potentially enhance haemocompatibility, while retaining the desirable properties for biomedical applications.

For understanding the potential of NTPDase attachment, NTPDase was immobilized on aminolyzed and carboxylated PET. Attachment of NTPDase was assessed by studying the activity of immobilized NTPDase. The effectiveness of NTPDase immobilization on aminolyzed and carboxylated PET was assessed by comparing the NTPDase activity of the above mentioned polymers with the activity observed on NTPDase immobilized on hydrolyzed PET (Marconi *et al.* 1983). Stability of NTPDase immobilization on these polymers was also studied.

Besides exploring the effects of three PET surface modification techniques, namely aminolysis, hydrolysis and carboxylation, this chapter also analyzes the immobilization of NTPDase to aminolyzed and carboxylated PET. By performing these tasks, this chapter analyzes both aspects involved in developing a useful biomedical

material, namely, physical properties of the modified polymer and the ability to immobilize biologically active molecules to the modified surface.

3.2 Materials and methods

Polyethylene terephthalate (PET) (thickness = 0.2 mm) was supplied by DuPont (Hopewell, VA). Ethylenediamine, bromoacetic acid, 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC), 2-(N-morpholino)ethanesulfonic acid (MES), tris[hydroxymethyl]aminomethane (Trizma base), malachite green hydrochloride, ammonium molybdate tetrahydrate, polyoxyethylenesorbitan monolaurate (Tween 20), adenosine diphosphate (ADP) and NTPDase (Apyrase from potato, Grade VII containing the isoenzyme Desirée with low ATPase/ADPase ratio) were purchased from Sigma (St. Louis, MO). Sodium hydroxide, sodium phosphate (monobasic and dibasic), sodium chloride, acetone, acetic acid, formaldehyde, calcium chloride, sulfuric acid and other reagents were purchased from Fisher Scientific (Fair Lawn, NJ).

Before modifying the surfaces to introduce reactive functional groups, PET films, were cut in to 5 cm × 1 cm strips, soaked in acetone for 24 h and then dried in a nitrogen environment. These acetone-treated PET films were used in the following protocols.

3.2.1 Aminolysis

PET surfaces were aminolyzed by treatment with ethylenediamine using the protocol described earlier (Desai and Hubbell 1991). Briefly, 5 cm × 1 cm acetone-

treated PET was reacted with 5 ml of either 20, 40 or 60% ethylenediamine solution for 24 h at 40˚C (Gappa-Fahlenkamp and Lewis 2005). This formed aminolyzed PET by introducing primary amines to the ester linkage (Figure 3.1a). In addition to introducing primary amines, ethylenediamine treatment also exposes carboxylic acid functional groups on the PET surface due to the breaking of ester linkages (Figure 3.1b) (Aggarwal *et al.* 2005).

3.2.2 Hydrolysis

PET surfaces were hydrolyzed by performing an alkaline hydrolysis described earlier (Dave *et al.* 1987). Acetone treated PET was reacted with 5 ml of 1 M sodium hydroxide solution at 50˚C for 5 h. This cleaved the ester bonds present in PET exposing reactive carboxyl groups, referred to as hydrolyzed PET in this work (Figure 3.1c).

3.2.3 Carboxylation

PET surfaces were carboxylated using the protocol described earlier (Yang *et al.* 2000). Briefly, acetone treated PET was incubated with 18.5% formaldehyde solution in 1 M acetic acid for 4 h. This hydroxylated the PET surface (Figure 3.1d) (Massia and Hubbell 1990). The hydroxylated PET was then treated with 1 M bromoacetic acid solution in 2 M sodium hydroxide for 18 h (Löfås and Johnsson 1990) . This introduced the carboxyl group to the PET surface, referred to as carboxylated PET (Figure 3.1e).

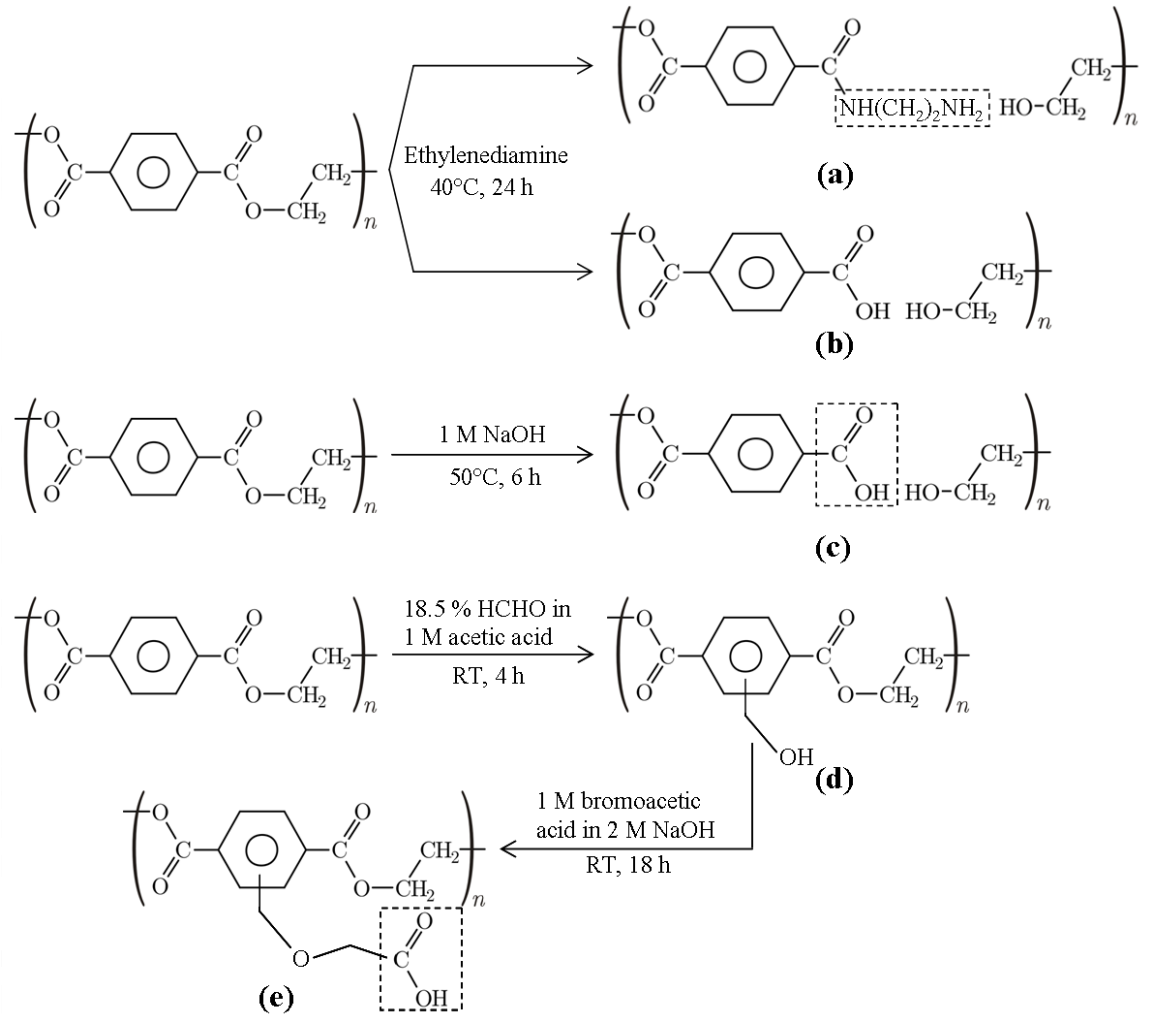


Figure 3.1. Schematic diagram showing surface modification of PET (a) primary amines introduced by ethylenediamine treatment, (b) carboxyl groups exposed due to aminolysis, (c) carboxyl groups introduced by hydrolysis, (d) hydroxylated intermediates formed during carboxylation and (e) carboxyl groups introduced by carboxylation. The active functional groups introduced to the PET surface are highlighted

All modified PET was thoroughly washed three times with DI water and soaked in DI water for 5 minutes. This was repeated three times. Then, modified PET was dried in a glove box under a nitrogen environment before being used.

3.2.4 Analysis of surface properties

Scanning electron microscope (SEM) studies were performed to verify possible PET surface deterioration due to the modifications. The SEM was used to obtain high resolution images that provided a visual perspective of the three dimensional quality of the surface being studied. SEM uses a high energy electron beam that focuses and scans the surface to be studied. As the high energy beam hits the surface, low energy secondary electron emissions are emitted from the spot. The intensity of the low energy secondary electrons is a function of the atomic composition and the geometry of the surface being studied. The intensity of the secondary electron emission is used to spatially reconstruct the surface image on a phosphor screen or a charged coupled device detector. Since the secondary electrons emitted have low penetration, only the electrons emitted from the surface escape and impact the intensity. Hence, SEM images are used for surface analysis. In the case of non-conductive materials, a thin metal coating is given to the surface to reduce accumulation of negative charges from the electron beam. For this research, modified and unmodified PET surfaces were coated with a thin layer of gold and SEM images were captured using a Jeol JSM 6360 scanning electron microscope (Jeol USA, Inc., Peabody, MA).

Atomic force microscopy (AFM) studies were performed to measure the roughness factors of modified and unmodified PET surfaces. AFM uses a piezo drive mechanism, which measures the deflection of a tip mounted on a flexible cantilever arm. The tip is deflected due to van der Waals and electrostatic repulsion and attraction between an atom at the tip and an atom on the surface. The movements of the cantilever arm are magnified by monitoring the position of a laser, which is reflected by a mirror on the cantilever arm, on a spatially resolved photosensitive detector. AFM is used to study surfaces using primarily one of the two modes, namely, contact mode and tapping mode. In contact mode, the tip is in contact with the surface. The contact mode is effective in studying the surface of rigid materials. However, in the case of softer specimens, like proteins and polymers, the surface could be damaged by the contact mode AFM studies. In the case of tapping mode or intermittent force mode, the tip barely touches the surface. The tip oscillates at a frequency close to the resonance of the cantilever. The force interaction of the tip and the surface can affect the amplitude of the oscillation and the oscillation frequency of the tip. This change is the amplitude is used to measure the surface properties. In this study, the roughness factors of modified and unmodified PET surfaces were measured using a Veeco Multimode Scanning Probe Microscope with conductive AFM module and Nanoscope V controller (Veeco Metrology Inc., Tucson, AZ) operated in a tapping mode.

3.2.5 Analysis of mechanical properties

Tensile testing of modified and unmodified PET was performed by using an Instron 5542 single column testing system (Instron, Grove City, PA). PET films were cut into 5 cm × 1 cm strips. These strips were loaded onto the testing system and were tightly clamped to avoid any slippage. A tensile stress was then applied at 10 mm of elongation per minute. The strain on the PET strip was measured. Elongation was continued until the PET film ruptured. The maximum load applied, tensile stress and strain at break, and the Young's modulus were computed using the data obtained.

3.2.6 NTPDase immobilization

Aminolyzed PET was incubated with 20 U/ml of NTPDase at 4˚C for 16 h. NTPDase is immobilized on aminolyzed PET using imine bonds (similar to studies in the literature (Brown *et al.* 1968; Patel *et al.* 1969)) between the primary amine group on the aminolyzed PET and possibly the aldehyde groups, which are introduced by glycosylation during posttranslational modification of the glycoprotein NTPDase (Ivanenkov *et al.* 2005). However, if mannose exists as a hemi-acetal in the ring structure causing the aldehyde groups to be inaccessible, NTPDase immobilization could have occurred due to adsorption. This modified polymer is denoted as PET-NH_2-NTPDase in this work (Figure 3.2a). Since the reaction is carried out in the absence of any carboxyl group activating agent, NTPDase doesn't form an amide bond with the exposed carboxyl groups on the aminolyzed PET (Figure 3.1b).

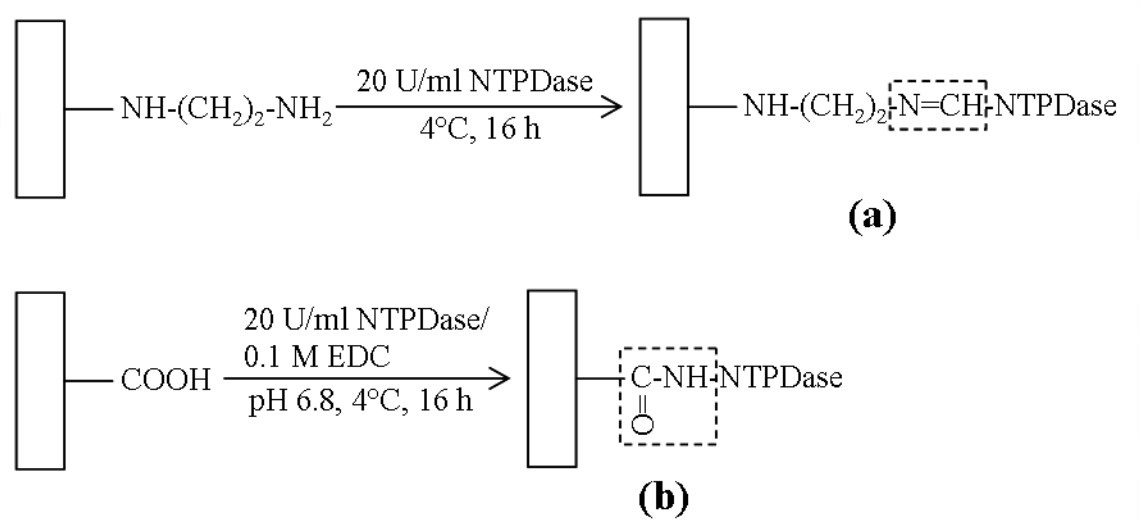


Figure 3.2. Schematic diagram showing NTPDase immobilization: (a) NTPDase immobilized on aminolyzed PET via imine bonds [PET-NH_2-NTPDase], (b) NTPDase immobilized on carboxylated PET via amide bonds [PET-COOH-NTPDase]

Carboxylated PET was incubated with 0.1 M EDC and 20 U/ml of NTPDase in 0.1 M phosphate buffer (pH = 6.8) at 4˚C for 16 h. This immobilized NTPDase on carboxylated PET using amide bonds between the carboxyl group on the carboxylated PET and ε-amine group of the lysine molecules on NTPDase (Marconi *et al.* 1983). This modified polymer is denoted as PET-COOH-NTPDase in this work (Figure 3.2b). NTPDase was also immobilized using 0.1 M MES buffer (pH = 6.8) instead of phosphate buffer.

Control samples, to confirm the absence of NTPDase immobilization and/or absorption without PET modification, were prepared by incubating acetone treated PET with 20 U/ml of NTPDase at 4ºC for 16 h. All the modified polymers and control samples were first washed using 1 M NaCl and then thoroughly rinsed with DI water.

3.2.7 Analysis of NTPDase attachment

The attachment of NTPDase was verified by exposing 10 cm^2 of NTPDase modified PET to 50 ml of 0.25 mM ADP in the presence of 0.1 M Tris buffer (pH = 7.4) and 5 mM $CaCl_2$ (Figure 3.3a). ADP was converted to AMP and inorganic phosphate (Pi). The sample solution was assayed for Pi concentration using a malachite green solution assay (Baykov *et al.* 1988; Geladopoulos *et al.* 1991). Briefly, 2.5 ml of 7.5% ammonium molybdate, 10 ml of 0.122% malachite green in 6 N H_2SO_4 and 0.2 ml of 0.11% Tween 20 were mixed to form the malachite green solution. 800 µl of sample was mixed with 200 µl of the malachite green solution.

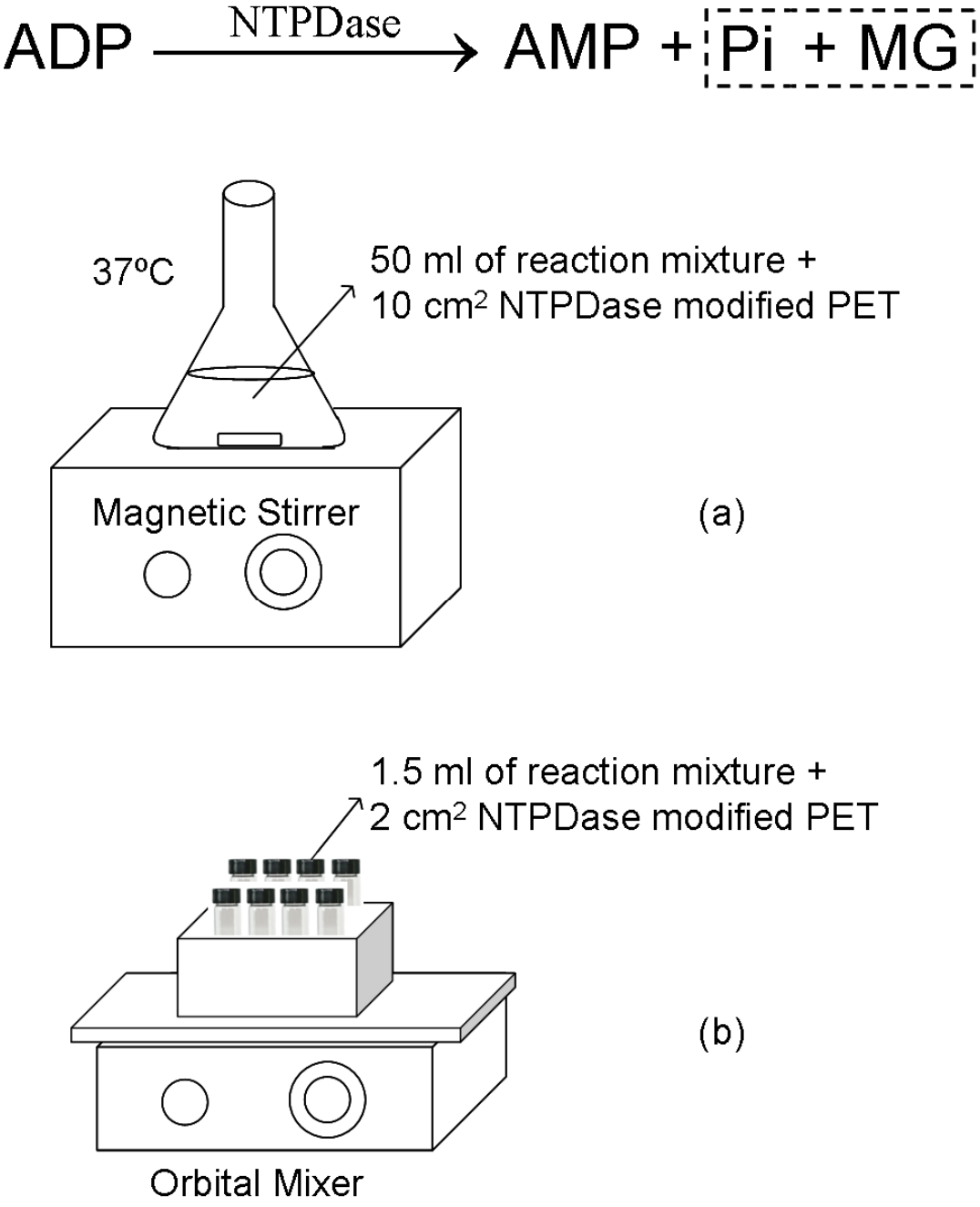


Figure 3.3. Experimental designs for analyzing NTPDase attachment. (a) 50 ml of 0.25 mM ADP in the presence of 0.1 M Tris buffer (pH = 7.4) and 5 mM $CaCl_2$ with 10 cm^2 of NTPDase modified PET (b) 1.5 ml of 0.25 mM ADP in the presence of 0.1 M Tris buffer (pH = 7.4) and 5 mM $CaCl_2$ with 2 cm^2 of NTPDase modified PET. Inorganic phosphate (Pi) concentration is measured using a malachite green assay (MG).

Ammonium molybdate reacts with the inorganic phosphate to form phosphomolybdate. This phosphomolybdate forms a highly colored complex with malachite green, which is stabilized by Tween 20. The absorbance was measured at 630 nm using a Shimadzu UV-1601 UV-visible spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD). Similar experiments were also performed using control samples to verify covalent attachment of NTPDase.

Malachite green solution (MG) was prepared freshly on the day of the experiments. As multiple experiments were performed on the same day, it was important to verify if the assay predicted the Pi concentration consistently when the MG was stored for prolonged hours. To confirm this, MG reagent was prepared. The freshly prepared reagent was first calibrated using phosphate standards prepared with potassium phosphate (tribasic). 800 µl of standard and 200 µl of MG solution were mixed and allowed to stand for 10 minutes and the absorbance was measured at 630 nm. Similar calibrations were performed after storing the MG solution for 3, 6, 9 and 11 hours. The results, shown in Figure 3.4, clearly prove that the assay is stable over the period of time. Therefore, MG solution was prepared before running all the experiments.

For some experiments involving PET-COOH-NTPDase, 2 cm^2 of PET-COOH-NTPDase was exposed to 1.5 ml of 0.25 mM ADP in the presence of 0.1 M Tris buffer (pH = 7.4) and 5 mM $CaCl_2$ (Figure 3.3b). To study the stability of immobilizations, NTPDase modified PET was first stored in air or in solution containing equal volumes of 5 mM $CaCl_2$ and 0.1 M Tris buffer (pH = 7.4) for 100 or 200 min. Then, the stored polymers were washed thoroughly with DI water before performing the analysis. All analysis and storage was at 37˚C.

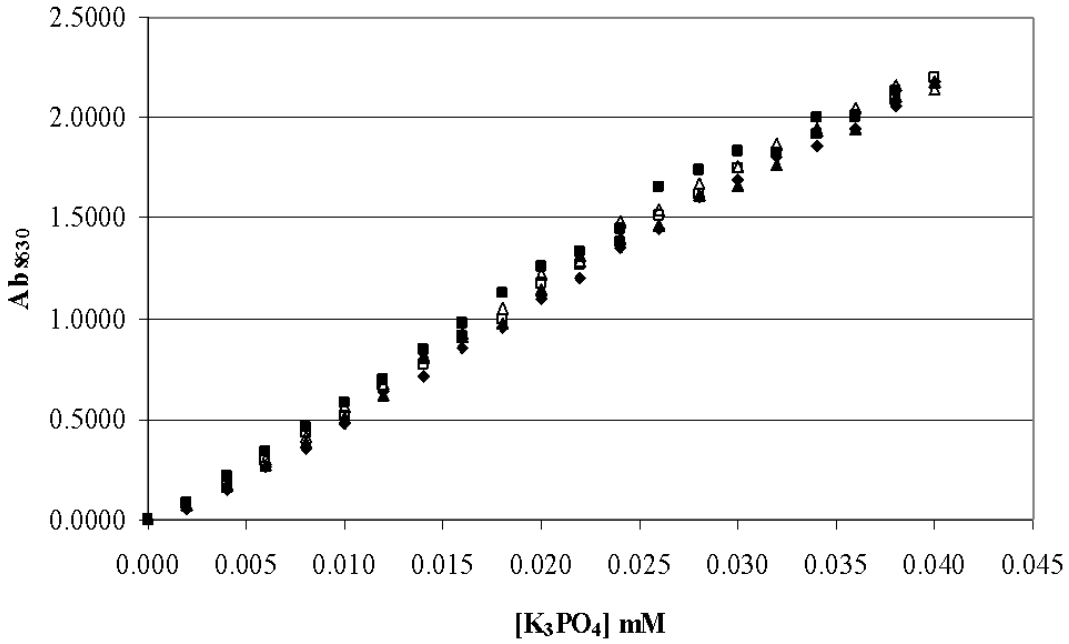


Figure 3.4. Malachite green assay sensitivity over time. The plot shows absorbance at 630 nm (Abs_{630}) vs. Pi concentrations for malachite green solution that was freshly prepared (♦), stored for 3 h (□), 6 h (▲), 9 h (Δ) and 11 h (■).

3.3 Results and Discussion

3.3.1 Surface characterization of modified PET

SEM studies were performed to analyze the possibility of surface deterioration due to chemical modifications. Figure 3.5a is an SEM image of unmodified PET. Unmodified PET shows a smooth surface with no deterioration. However, as seen in Figure 3.5b, aminolysis resulted in the formation of cracks on the surface. Aminolyzed PET showed significantly different surface properties compared to the unmodified PET. Figure 3.5c highlights pit formation on hydrolyzed PET surfaces. These pits, formed due to the ester bond breakage during alkaline hydrolysis, alter the surface properties of the hydrolyzed PET significantly. These images are consistent with previous studies that have described cracks due to aminolysis (Ellison *et al.* 1982; Holmes 1996; Nissen *et al.* 2008) and pitting due to hydrolysis (Ellison *et al.* 1982; Rahman and East 2006).

On the other hand, carboxylated PET (Figure 3.5d) does not show any cracks or pits that would modify the surface properties and potentially weaken the mechanical strength. The spots seen on the surface of carboxylated PET were not caused by surface modifications as similar spots were also observed on SEM images of several unmodified PET samples. Thus, surface properties were not significantly altered due to carboxylation, while they were drastically changed by aminolysis and hydrolysis. Although the qualitative analysis using SEM images shows that carboxylated PET would show surface properties similar to unmodified PET, it was also important to perform a quantitative analysis.

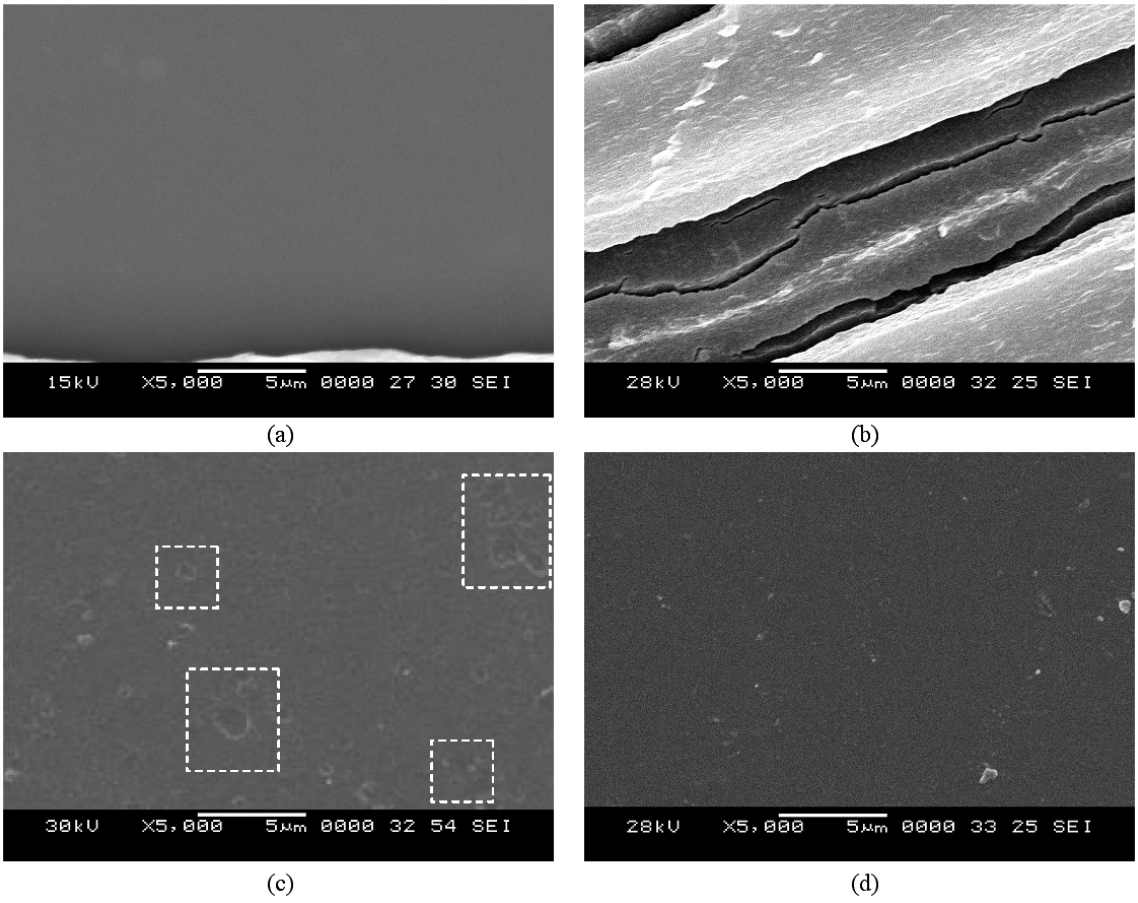


Figure 3.5. Scanning Electron Microscope images of (a) unmodified PET, (b) aminolyzed PET, (c) hydrolyzed PET and (d) carboxylated PET. Cracks on aminolyzed PET are clearly visible and pits on hydrolyzed PET are highlighted.

To further understand the change in surface properties quantitatively, AFM studies were performed. Roughness factors of unmodified, aminolyzed, hydrolyzed and carboxylated PET were measured using the protocol described earlier. The results are shown in Table 3.1. Unmodified PET was very smooth with a low roughness factor (Figure 3.6a). While preparing the aminolyzed PET using 60% ethylenediamine for aminolysis, the roughness factor increased drastically. The high roughness factor couldn't be measured using an AFM. Hence, AFM studies were performed with aminolyzed PET using a 20% and 40% ethylenediamine solution (Figures 3.6b and 3.6c respectively). The results show that the roughness of aminolyzed PET increased with an increase in the concentration of ethylenediamine used during aminolysis. Based on this, it is clear that using 60% ethylenediamine for introducing amine groups on PET surfaces would severely alter the surface properties. The average roughness factor of hydrolyzed PET was almost twice that of unmodified PET. However, the regions on hydrolyzed PET that showed pitting (as highlighted in Figure 3.6d) had roughness factors as high as 37.9 nm. These AFM studies confirm the observations from SEM images about pitting due to hydrolysis of PET.

On the other hand, carboxylated PET showed a similar roughness as unmodified PET (Figure 3.6e). These studies show that carboxylated PET shows no surface deterioration and minimal surface roughness, comparable to unmodified PET. Based on these results, it is evident that carboxylation of PET to introduce reactive functional groups to the surface is clearly more desirable compared to aminolysis and hydrolysis.

Table 3.1. Roughness analysis of modified polymers

Polymer	**Roughness factor (nm)**
Unmodified	4.94±2.41
20% Aminolyzed	21.87±4.66
40% Aminolyzed	109.00
Hydrolyzed	11.32±3.22
Carboxylated	4.17±1.40

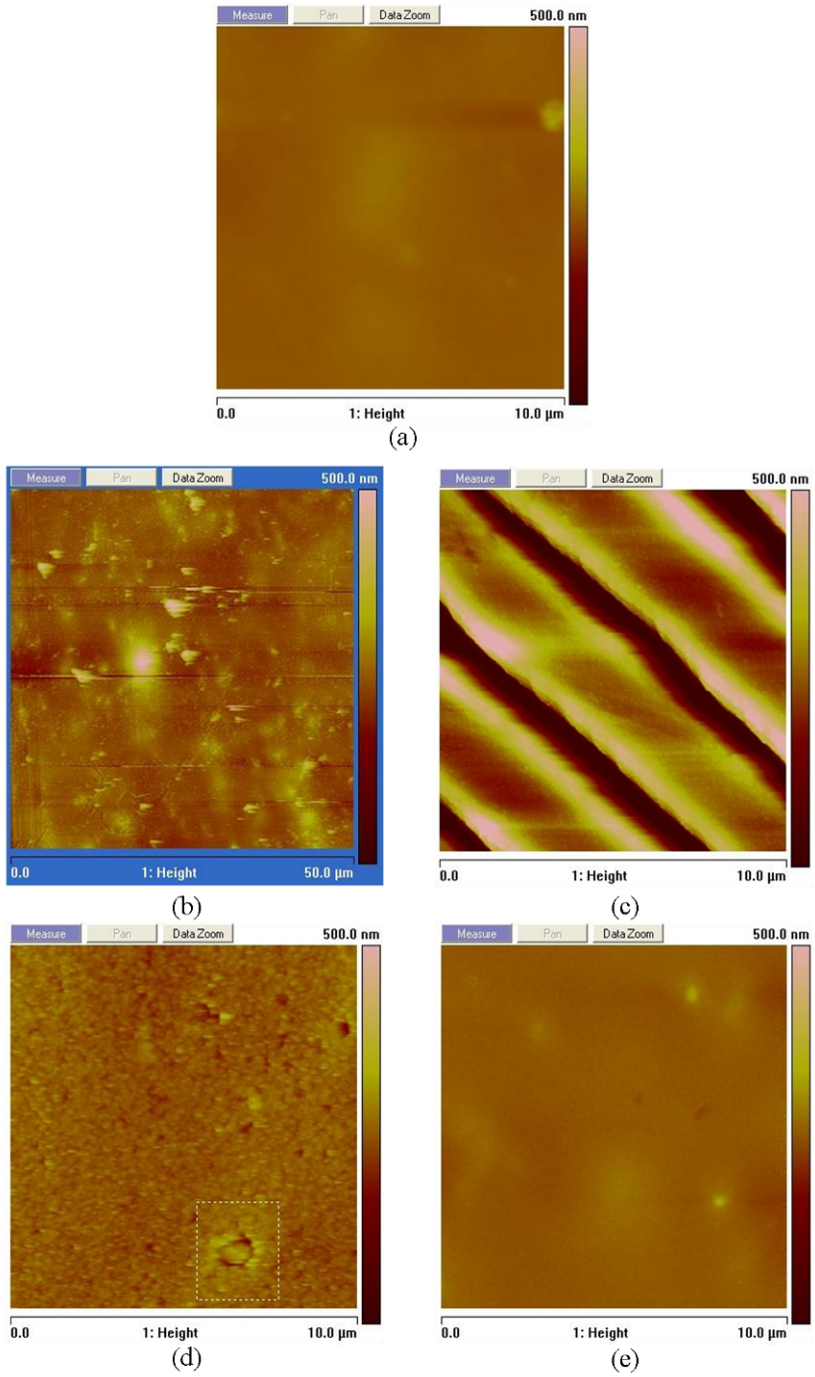


Figure 3.6. Atomic Force Microscope images of (a) unmodified PET, (b) 20% aminolyzed PET, (b) 40% aminolyzed PET, (d) hydrolyzed PET and (e) carboxylated PET. Cracks on aminolyzed PET are clearly visible and pits on hydrolyzed PET are highlighted.

3.3.2 Bulk characterization of modified PET

Based on the surface characterization of unmodified, aminolyzed, hydrolyzed and carboxylated PET, it was expected that aminolyzed and hydrolyzed PET would show lesser mechanical strength due to the surface cracks and pits. To verify this, mechanical strengths of modified and unmodified PET were compared by tensile testing. As expected, the results, shown in Table 3.2, confirm significant loss of tensile strength in aminolyzed and hydrolyzed PET due to the cracks and pits formed. This is consistent with previous studies (Ellison *et al.* 1982; Kao *et al.* 1998). In contrast, the results show that carboxylated PET exhibits a tensile strength comparable to unmodified PET. It is clear that carboxylated PET shows more desirable surface and bulk properties compared to aminolyzed and hydrolyzed PET.

3.3.3 NTPDase immobilization on aminolyzed PET

As described earlier, NTPDase was immobilized on aminolyzed PET (PET-NH_2-NTPDase), possibly by imine bonds between the primary amines on aminolyzed PET and the aldehyde groups present on the glycoprotein NTPDase. The presence of active NTPDase on aminolyzed PET was tested by performing NTPDase kinetic experiments in 50 ml of 0.25 mM ADP (12.5 μmoles) exposed to 10 cm^2 of PET-NH_2-NTPDase. Figure 3.7 shows that freshly prepared PET-NH_2-NTPDase had significant NTPDase activity and that all of the ADP released Pi within approximately 30 minutes. The initial release rate of Pi is approximately 3 μmoles Pi released h^{-1} cm^{-2}.

Table 3.2. Tensile testing of modified polymers

Polymer	**Max Load (N)**	**Tensile Stress at Break (MPa)**	**Tensile Strain at Break (MPa)**	**E-modulus (MPa)**
Unmodified	100	197	71	3700 ± 200
60% Aminolyzed	65	49	36	3450 ± 600
Hydrolyzed	72	48	31	3150 ± 300
Carboxylated	103	205	77	3350 ± 150

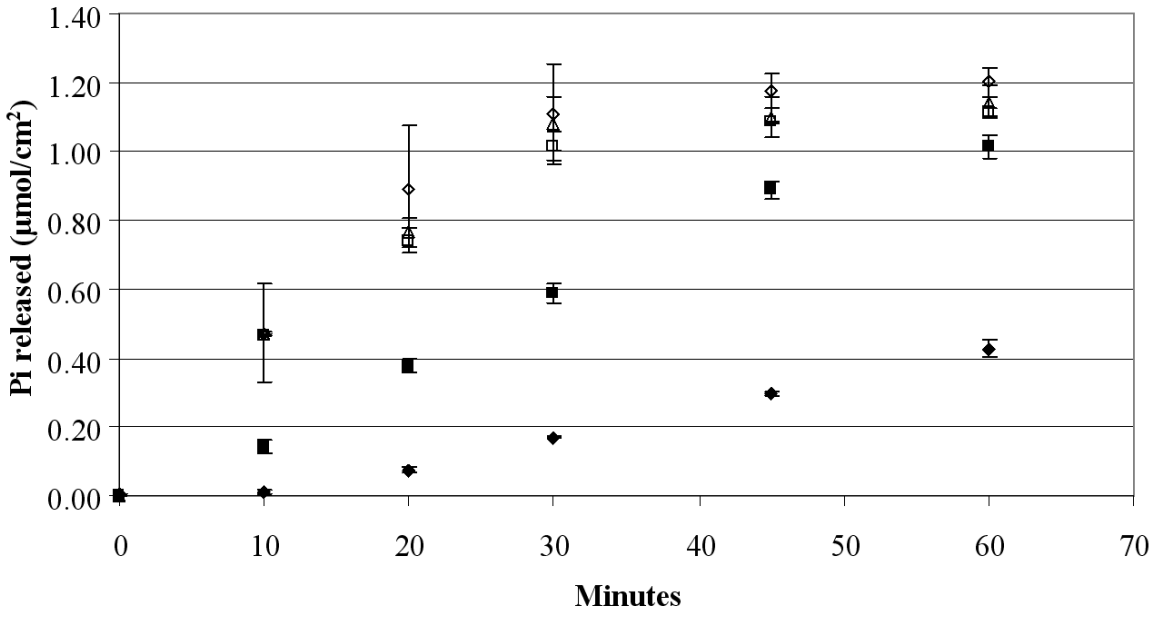


Figure 3.7. Rate of inorganic phosphate (Pi) release due to ADP hydrolysis catalyzed by NTPDase immobilized on aminolyzed PET (PET-NH_2-NTPDase). Pi released from 50 ml of 0.25 mM ADP (initially 12.5 µmoles) by 10 cm^2 freshly prepared (◊), stored in air for 100 min (□) stored in air for 200 min (Δ), stored in solution for 100 min (■), and stored in solution for 200 min (♦) are shown. The solution in which PET-NH_2-NTPDase was stored contains equal volumes of 0.1 M Tris buffer (pH = 7.4) and 5 mM $CaCl_2$. All storage and kinetic analysis were done at 37˚C.

PET-NH_2-NTPDase was also stored at 37˚C in air and in a solution containing equal volumes of 0.1 M Tris buffer (pH = 7.4) and 5 mM $CaCl_2$ solution prior to kinetic analysis. PET-NH_2-NTPDase was removed after 100 and 200 minutes in storage conditions and tested for activity. Figure 3.7 shows that the rate and amount of Pi released due to prior storage in air for 100 min and 200 min was comparable to that of freshly prepared PET-NH_2-NTPDase. This indicates no loss of NTPDase activity with time when stored in air. Figure 3.7 also shows that the rate and amount of Pi released due to prior storage in solution was significantly lower for 100 minutes of storage and even more significantly lower for 200 minutes than that of freshly prepared PET-NH_2-NTPDase. This was probably due to loss of NTPDase into the storage solution. The storage solution showed detectable NTPDase activity. However, the activity could not be quantified reliably. This problem can potentially be overcome by reducing the imine bonds into an alkylamine linkage (Hermanson 1996). Since the aminolyzed PET had mechanical strength limitations, stabilizing the immobilization was not explored.

3.3.4 NTPDase immobilization on carboxylated PET

NTPDase was also immobilized to carboxylated PET (PET-COOH-NTPDase) by an amide bond between the carboxyl group on the polymer and the ε-amine group of the lysine molecule on NTPDase. When 10 cm^2 of freshly prepared PET-COOH-NTPDase was exposed to 50 ml of a 0.25 mM ADP solution (12.5 μmoles), ADP was not quickly consumed to form an equivalent amount of Pi– unlike PET-NH_2-NTPDase which

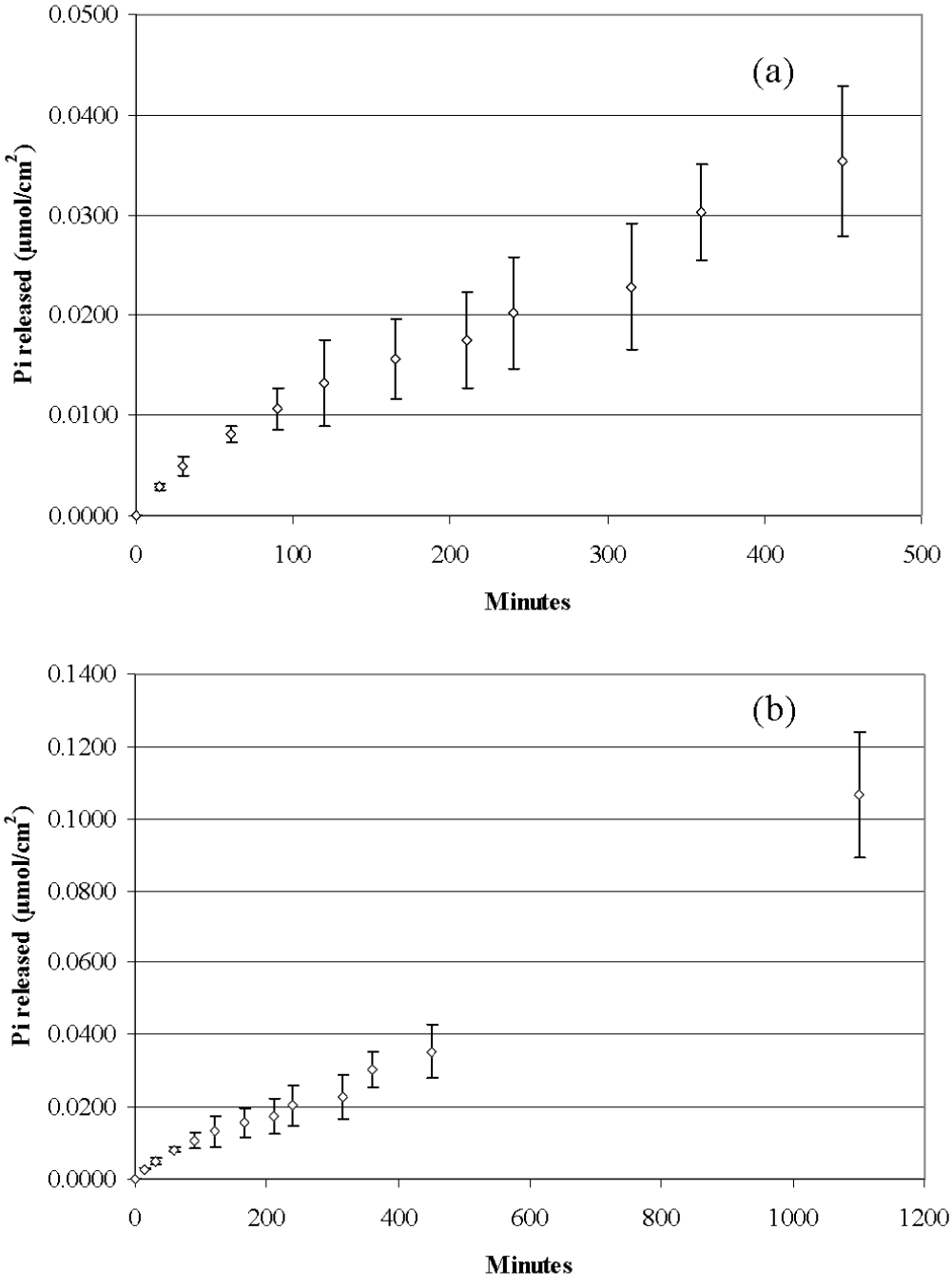


Figure 3.8. Inorganic phosphate (Pi) release due to ADP hydrolysis catalyzed by NTPDase immobilized on carboxylated PET (PET-COOH-NTPDase) for prolonged reaction. Pi released from 50 ml of 0.25 mM ADP (initially 12.5 µmoles) by 10 cm^2 of PET-COOH-NTPDase (a) during the first 450 minutes and (b) after 18 hours are shown.

completely consumed the ADP within 60 minutes. However, when the reaction continued for over 18 h, ADP was mostly consumed to form Pi in an amount similar to the initial ADP concentration (Figure 3.8b).

The stability of PET-COOH-NTPDase was then studied by storing PET-COOH-NTPDase at 37˚C in air and in a solution containing equal volumes of 0.1 M Tris buffer (pH = 7.4) and 5 mM $CaCl_2$ solution for 200 min before analysis. 10 cm^2 of these stored PET-COOH-NTPDase polymers were exposed to 50 ml of 0.25 mM ADP (12.5 μmoles), similar to the experimental conditions used for Figure 3.8. Figure 3.9 shows that the amount of Pi released with prior storage in air or solution was comparable to that of freshly prepared PET-COOH-NTPDase. In contrast to PET-NH_2-NTPDase, these results clearly show that there was no detectable loss of NTPDase activity due to storage conditions. These experiments demonstrate a stable NTPDase immobilization on a mechanically strong PET surface.

All these studies were performed using PET-COOH-NTPDase prepared with 0.1 M phosphate buffer (pH = 6.8) during carbodiimide coupling. However, phosphate buffer is known to interfere with carbodiimide coupling to form a phosphoramidate linkage (Hermanson 1996). Hence, PET-COOH-NTPDase was also prepared in 0.1 M MES buffer (pH = 6.8) during carbodiimide coupling. 2 cm^2 of PET-COOH-NTPDase polymers prepared in the two different buffers were separately exposed to 1.5 ml of 0.25 mM ADP (0.375 μmoles). Figure 3.9 shows a very low rate of Pi release by PET-COOH-NTPDase prepared in MES buffer compared to that of PET-COOH-NTPDase prepared in phosphate buffer.

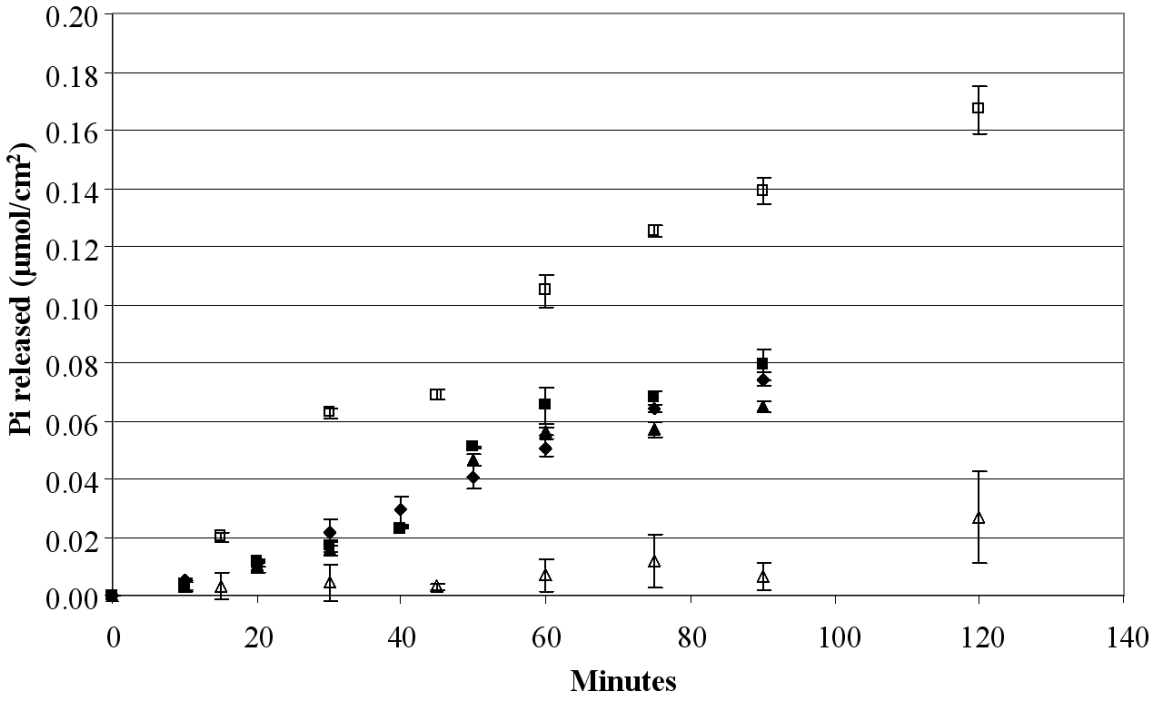


Figure 3.9. Inorganic phosphate (Pi) release due to ADP hydrolysis catalyzed by NTPDase immobilized on carboxylated PET (PET-COOH-NTPDase). Pi released from 50 ml of 0.25 mM ADP (initially 12.5 µmoles) by 10 cm^2 freshly prepared (♦), stored in air for 200 min (▲), stored in solution for 200 min (■) are shown. All PET-COOH-NTPDase were prepared in phosphate buffer. The solution contains equal volumes of 0.1 M Tris buffer (pH = 7.4) and 5 mM $CaCl_2$. All storage was done at 37˚C. The figure also shows Pi released from 1.5 ml of 0.25 mM ADP (initially 0.375 µmoles) by 2 cm^2 PET-COOH-NTPDase freshly prepared in phosphate buffer (□) and freshly prepared in MES buffer (Δ). All storage and kinetic analysis was performed at 37˚C.

The initial release rate of Pi for PET-COOH-NTPDase prepared in phosphate buffer and exposed to 1.5 ml was approximately 0.13 μmoles Pi released h^{-1} cm^{-2}, which is 4% of the release rate for PET-NH_2-NTPDase. One possible reason for PET-NH_2-NTPDase showing a higher Pi release rate could be the presence of a higher NTPDase concentration on PET-NH_2-NTPDase. Since the available surface area for aminolyzed PET is greater than the external surface area due to surface cracks, more NTPDase could be attached to aminolyzed PET as compared to carboxylated PET. It is important to note that release rates were calculated based on the external surface area and that inclusion of the surface area due to cracks would reduce the calculated release rate of Pi for PET-NH_2-NTPDase.

In comparing Figures 3.8-3.9 for the 50 ml volume/10 cm^2 studies, it is notable that the amount of Pi released at a given time was much lower in Figure 3.8 as compared to Figure 3.9. Upon further examination, it should be noted that the data shown in Figure 3.8 was obtained using PET-COOH-NTPDase prepared with an old batch of NTPDase. Therefore, it's important to recognize the impact of the NTPDase source on making the polymers. Another important note is that in Figure 3.9, the release of Pi per unit membrane area is approximately 2x higher in the 1.5-ml reactor as compared to the 50-ml reactor. Furthermore, it was observed that almost the entire ADP was hydrolyzed to form Pi when PET-COOH-NTPDase (prepared with phosphate buffer) was exposed to a 1.5 ml solution as compared to a 50 ml solution.

The following mathematical analysis was used to assess the reason for the different Pi accumulation rates (per unit polymer area) observed for different liquid

volumes. Using a mole balance in a well-mixed bulk solution, the rate of accumulation of $\langle P_i \rangle$ (moles of phosphate per unit polymer area) in the bulk solution is given as

$$\frac{d\langle P_i \rangle}{dt} = k_L\left([ADP]_b - [ADP]_i\right) \tag{3.1}$$

where k_L is the mass transfer coefficient in length per unit time and $[ADP]_b$ and $[ADP]_i$ are the bulk and interfacial concentrations of ADP, respectively, in moles per unit volume. Using a mole balance at the polymer interface and assuming no accumulation in the adjacent boundary layer, the rate of accumulation of $\langle P_i \rangle$ is also given as

$$\frac{d\langle P_i \rangle}{dt} = \frac{V}{A}\frac{v_{\max}[ADP]_i}{K_M + [ADP]_i} \tag{3.2}$$

where v_{max} and K_M are Michaelis-Menten constants in moles per unit volume per unit time and moles per unit volume, respectively.

If mass transfer is the only limiting mechanism for ADP conversion to Pi in both 1.5-ml and 50-ml reactors, then $[ADP]_b >> [ADP]_i$. From equation 3.1, the rate of accumulation of $\langle P_i \rangle$ would become

$$\frac{d\langle P_i \rangle}{dt} = k_L[ADP]_b \tag{3.3}$$

Therefore, if both volume studies had the same initial ADP concentration and were mass transfer limiting with the same k_L, the rates of $\langle P_i \rangle$ accumulation would be the same. This is contrary to the experimental results. If k_L was higher for the lower volume study, this would result in a faster $\langle P_i \rangle$ accumulation rate, which is consistent with observations. It should be noted that in the 50-ml reactor, the polymer was cut into five 1 cm × 1 cm pieces and added to 50 ml of reaction mixture that was mixed using a magnetic stirrer. Under this experimental setup, the polymer pieces were continuously

moving in the reaction mixture. For the 1.5-ml reactor, a 1 cm × 1 cm polymer piece was introduced to a 2-ml vial containing 1.5 ml of reaction mixture that was mixed using an orbital mixer. The polymer piece was stationary inside the 2-ml vial as the liquid was moving. Under these conditions, the relative motion of the reaction mixture to the polymer would be higher in the 1.5-ml reactor as compared to the 50-ml reactor. Therefore, there is a strong possibility that k_L was higher in the 1.5-ml reactor as compared to the 50-ml reactor.

Another scenario is when both mass transfer and reaction kinetics play a role in regulating the rate for both 1.5-ml and 50-ml studies. This scenario can be examined by equating equations 3.1 and 3.2 such that:

$$\frac{k_L A}{V}\left([ADP]_b - [ADP]_i\right) = \frac{v_{\max}[ADP]_i}{K_M + [ADP]_i} \tag{3.4}$$

Rearrangement of equation 3.4 would show that $[ADP]_i$ increases with increasing k_LA/V. Therefore, when k_LA/V increases, the driving force ($[ADP]_b - [ADP]_i$) is lowered. For the 1.5-ml reactor in comparison to the 50-ml reactor, A/V is larger and it's likely that k_L is higher (see preceding paragraph)—leading to a larger k_LA/V. Also, v_{max} is a product of the enzyme rate constant and the enzyme concentration. Since the 50-ml reactor had 10 cm^2 polymer and the 1.5-ml reactor had 2 cm^2 polymer, v_{max} would be five times higher for the 50-ml reactor. Using this information in sensitivity analysis calculations, with assumed values of v_{max}, K_M and k_L, it can be shown that increasing k_LA/V can result in an increase (2x or more) in the rate of <Pi> accumulation. Again, this is consistent with the experimental observations.

Finally, a third scenario is when both 1.5-ml and 50-ml studies are not limited by mass transfer. In this scenario, the interfacial concentration of ADP is equal to the bulk concentration of ADP ($[ADP]_b=[ADP]_i$). Hence equation 3.2 becomes

$$\frac{d\langle P_i \rangle}{dt} = \frac{V}{A}\frac{v_{\max}[ADP]_b}{K_M + [ADP]_b} \tag{3.5}$$

Equation 3.5 shows that the rate of $<P_i>$ accumulation is proportional to the ratio of reaction volume to the area of polymer used. For the 50 ml studies, V/A= 5 and for the 1.5 ml studies, V/A = 0.75. Also, as noted above, v_{max} is a five times higher for the 50 ml study. Hence, if mass transfer doesn't play a role in both studies, the rate of $<P_i>$ accumulation would be significantly higher for the larger volume study which is contrary to the experimental results.

Based on the above analyses, if both studies have the same limiting conditions, the possible reason for the higher rate of <Pi> accumulation in the 1.5-ml reactor is a higher k_L due to the differences in hydrodynamic mixing conditions. It is also possible that 1.5-ml and 50-ml reactions have different limiting conditions. Considering the reactor mixing conditions in which flow relative to the polymer was likely higher with the 1.5-ml reactor, it's likely that the k_L value was higher. In addition, A/V is also higher for the 1.5-ml reactor. A higher k_LA/V would lead to a higher $[ADP]_i$ (see Equation 3.4) such that reaction kinetics would play a more dominant role. Hence, it is possible that the 1.5-ml reactor is kinetic-limited while the 50-ml reactor is mass transfer-limited. Such a condition would also result in an increased rate of <Pi> accumulation in the 1.5-ml reactor. Therefore, several possibilities discussed above would explain the higher rate of <Pi> accumulation in the 1.5-ml reactor.

3.3.5 Effect of EDC to NTPDase ratio on NTPDase immobilization

During NTPDase immobilization on carboxylated PET, the ratio of EDC to NTPDase was a critical factor. NTPDase activity on PET-COOH-NTPDase was significantly altered when a different ratio of EDC to NTPDase was used during immobilization. For understanding the importance of the EDC concentration on immobilization, PET-COOH-NTPDase was prepared using different concentrations of EDC (0.05 M, 0.1 M, 0.2 M, 0.3 M). The activity of NTPDase was studied. Although using higher concentrations of EDC helped in activating the carboxyl groups on the polymer surface, results showed that higher ratios of EDC concentration to NTPDase concentration resulted in a lower NTPDase activity (Figure 3.10). This is possibly because carboxyl groups present in NTPDase molecules also get activated, thereby resulting in reactions amongst NTPDase molecules themselves. This could result in loss of NTPDase activity.

3.3.6 Comparison of NTPDase immobilizations

From the results shown in Figures 3.7 and 3.9, it is clear that both PET-NH_2-NTPDase and PET-COOH-NTPDase show detectable levels of NTPDase activity. Experiments performed with the control samples didn't show any detectable NTPDase activity. This indicates that the NTPDase activity on the modified polymers is due to NTPDase immobilization.

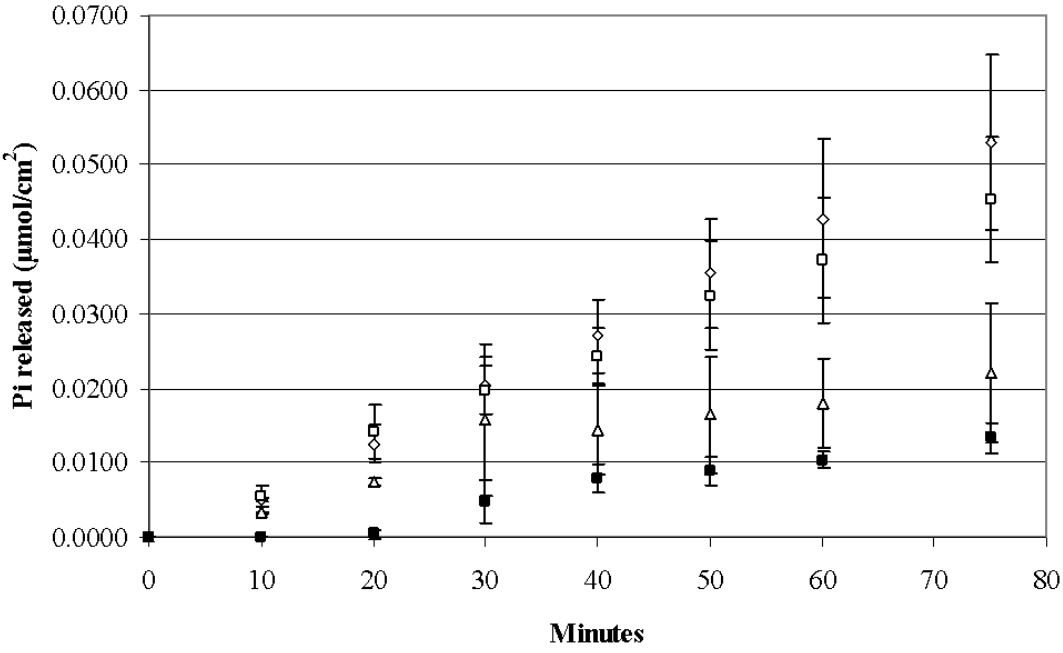


Figure 3.10. Rate of inorganic phosphate (Pi) release due to ADP hydrolysis catalyzed by NTPDase immobilized on carboxylated PET (PET-COOH-NTPDase) using different concentrations of EDC. Pi released from 50 ml of 0.25 mM ADP (initially 12.5 µmoles) by 10 cm^2 of PET-COOH-NTPDase prepared using 0.05 M EDC (◊), 0.1 M EDC (□), 0.2 M EDC (Δ) and 0.3 M EDC (■) are shown.

NTPDase activity on PET-NH_2-NTPDase was approximately twice the NTPDase activity achieved by Marconi et al. by immobilizing NTPDase on hydrolyzed PET (1.5 μmoles Pi released h^{-1} cm^{-2}) (Marconi *et al.* 1983). As discussed earlier, similar to aminolyzed PET, hydrolyzed PET also shows surface deterioration and loss of mechanical strength and would not be a viable polymer for biomedical applications. Although NTPDase activity on PET-NH_2-NTPDase was high, aminolyzed PET showed surface deterioration and a loss of mechanical strength. In addition, the immobilization was not stable in solution. However, the imine bonds present in PET-NH_2-NTPDase could be stabilized by reduction. As aminolyzed PET would not be a desirable choice for biomedical applications, studies were not performed to stabilize imine bonds in PET-NH_2-NTPDase.

In contrast, NTPDase immobilization was stable with carboxylated PET in that no surface deterioration was shown and mechanical strength was maintained. Although the NTPDase activity observed was 9% of the release rate observed with NTPDase immobilized on hydrolyzed PET (Marconi *et al.* 1983), it is important to recognize that a higher activity can be observed as a result of cracks or pits increasing the surface area for NTPDase attachment. Studies done with immobilizing NTPDase on nylon and C 50 steel rings showed a NTPDase activity around 0.3 μmoles Pi released h^{-1} cm^{-2}, which was sufficient to significantly improve platelet inhibition (Marconi *et al.* 1983). This activity is more consistent with the PET-COOH-NTPDase. Optimization of NTPDase immobilization on carboxylated PET could potentially improve the NTPDase surface concentration (and thus increase Pi release rate) of PET-COOH-NTPDase. Thus, carboxylated PET is a potentially viable polymer for stable NTPDase immobilization in

which significant NTPDase activity is present and the polymer maintains characteristics similar to unmodified PET.

3.4 Conclusions

In this study, both effects of PET surface modification and immobilization of NTPDase were analyzed. The effects of PET modifications, namely aminolysis, hydrolysis and carboxylation, on surface and bulk properties were studied by analyzing possible surface deterioration, roughness and mechanical strength of modified and unmodified PET. The results show that carboxylated PET has the minimum change in the surface and bulk properties as it maintained the roughness and mechanical strength of unmodified PET. Based on these results, carboxylated PET could potentially improve the biomedical applications of PET. Immobilization of biologically active molecules was studied by attaching NTPDase to aminolyzed and carboxylated PET. NTPDase could potentially improve the haemocompatibility of PET. NTPDase was successfully immobilized to aminolyzed PET and carboxylated PET using imine and amide bonds, respectively. NTPDase activity was significantly higher in PET-NH_2-NTPDase compared to PET-COOH-NTPDase. However, PET-NH_2-NTPDase exhibited imine bond instability in solution while PET-COOH-NTPDase showed stability in solution. The PET-COOH-NTPDase polymer also exhibited NTPDase activity that could potentially enhance platelet inhibition. Thus, a mechanically strong NTPDase-modified PET (PET-COOH-NTPDase) that showed bond stability in solution was demonstrated. Hence, it is important that the stability of immobilization is analyzed in different storage

environments when a molecule is attached to the modified polymer before performing further studies. In conclusion, carboxylation of PET showed great potential for biomedical applications due to minimum changes in surface and bulk properties and the ability to immobilize bioactive agents such as NTPDase.

CHAPTER 4

KINETIC STUDIES OF FREE AND IMMOBILIZED NTPDase

Studying both free and immobilized NTPDase kinetics would help in better understanding the mechanism of NTPDase functionality following immobilization. Such an understanding could be a valuable tool in optimizing the activity of NTPDase for obtaining improved haemocompatibility. With successful immobilization of NTPDase to a mechanically stable polymer, understanding the kinetics would add another dimension to developing a haemocompatible biomaterial. For this purpose, the kinetics of immobilized NTPDase were studied for PET-NH_2-NTPDase and PET-COOH-NTPDase. These results were compared with free NTPDase kinetics. As PET-COOH-NTPDase would potentially be used in blood-contact systems, it is important to study the effect of immobilization on NTPDase activity when the polymer is stored in a solution at 37ºC. For this purpose, the effect storing free NTPDase in a solution at 37ºC was studied and compared to the results discussed in Chapter 3 in which immobilized NTPDase was stored in a solution at 37ºC.

4.1 Introduction

NTPDase (EC 3.6.1.5) is an enzyme that catalyzes the hydrolysis of pyrophosphoric bonds in organic and inorganic compounds in the presence of a bivalent

cation. NTPDase activity has been reported in both animals and plants (Treuheit *et al.* 1992; Stout and Kirley 1994; Handa and Guidotti 1996; Basanova *et al.* 2002; Failer *et al.* 2002). In humans, NTPDase plays an important role in regulating blood coagulation (Gayle III *et al.* 1998; Weksler 2000). NTPDase hydrolyzes ADP released from platelets during blood coagulation, thereby attenuating platelet aggregation caused by ADP. Understanding the mechanism and kinetics involved in ADP hydrolysis by NTPDase would provide valuable information. As the goal of this research is to develop a haemocompatible polymer using NTPDase modification, studying NTPDase kinetics and the effect of immobilization on the kinetics was important. For this purpose, ADP hydrolysis by NTPDase-modified polymers (PET-NH_2-NTPDase and PET-COOH-NTPDase) was studied under physiological temperature and pH.

4.2 Materials and methods

Polyethylene terephthalate (PET) (thickness = 0.2 mm) was supplied by DuPont (Hopewell, VA). Ethylenediamine, bromoacetic acid, 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC), tris[hydroxymethyl]aminomethane (Trizma base), malachite green hydrochloride, ammonium molybdate tetrahydrate, polyvinyl alcohol (PVA), polyoxyethylenesorbitan monolaurate (Tween 20), adenosine diphosphate (ADP) and NTPDase (Apyrase from potato, Grade VII containing the isoenzyme Desirée with low ATPase/ADPase ratio) were purchased from Sigma (St. Louis, MO). Sodium hydroxide, sodium phosphate (monobasic and dibasic), sodium chloride, acetone, acetic

acid, formaldehyde, calcium chloride, sulfuric acid, hydrochloric acid and other reagents were purchased from Fisher Scientific (Fair Lawn, NJ).

4.2.1 Free NTPDase batch kinetics

For studying free NTPDase batch kinetics, 1 U of free NTPDase was added to 50 ml of reaction mixture containing ADP in the presence of 0.1 M Tris buffer (pH = 7.4) and 5 mM $CaCl_2$ at 37ºC. ADP was converted to AMP and inorganic phosphate (Pi). The Pi concentration was measured using a malachite green assay previously described (Chan *et al.* 1986; Henz *et al.* 2007). Briefly, 2.86 g ammonium molybdate was dissolved in 50 ml of 6 N HCl, 1.16 g PVA was dissolved in 50 ml boiling water and 0.0812 g of malachite green was dissolved in 100 ml DI water. The colorimetric reagent was prepared by mixing two parts of malachite green solution, one part each of ammonium molybdate and PVA solutions and two parts of DI water. This mixture was prepared on the day of use. 800 µl of sample solution was mixed with 200 µl of malachite green reagent and allowed to stand for 10 minutes. The absorbance was measured at 630 nm. Malachite green assay described by Baykov et al. was not used for free enzyme analysis, as free NTPDase interfered with the assay. The procedure was performed for different ADP concentrations (0.05, 0.1, 0.15, 0.2, 0.25 and 0.3 mM).

To assess the effect of storing free NTPDase in solution at 37ºC, NTPDase was dissolved in a mixture containing 0.1 M Tris buffer (pH = 7.4) and 5 mM $CaCl_2$. This NTPDase solution was stored at 37ºC for 60, 120 and 240 minutes. 0.25 mM ADP

solution was reacted with the stored NTPDase solution. The rate of Pi formation was compared for freshly prepared NTPDase solution and the stored NTPDase solutions.

4.2.2 Immobilized NTPDase batch kinetics

For studying immobilized NTPDase kinetics, PET-NH_2-NTPDase and PET-COOH-NTPDase polymers were used. PET-NH_2-NTPDase was prepared using the protocol described in Chapter 3. Briefly, aminolyzed PET, which was prepared by treating the polymer with 60% ethylenediamine at 40ºC for 24 h (Desai and Hubbell 1991), was incubated with 20 U/ml NTPDase solution at 4ºC for 16 h. PET-COOH-NTPDase was also prepared using the protocol described in Chapter 3. Briefly, PET was first hydroxylated by reacting with 18.5% formaldehyde solution in 1 M acetic acid (Massia and Hubbell 1990). This hydroxylated PET was then carboxylated by reacting with 1 M bromoacetic acid in 2 M NaOH solution (Löfås and Johnsson 1990). Carboxylated PET was then treated with 0.1 M EDC and 20 U/ml NTPDase in 0.1 M phosphate buffer (pH = 6.8) to form PET-COOH-NTPDase.

Batch kinetics studies for PET-NH_2-NTPDase were performed exposing 10 cm^2 of PET-NH_2-NTPDase to 50 ml of ADP in the presence of 0.1 M Tris buffer (pH = 7.4) and 5 mM $CaCl_2$ at 37ºC. The rate of Pi release was measured using the malachite green assay described earlier (Baykov *et al.* 1988). Briefly, 2.5 ml of 7.5% ammonium molybdate, 10 ml of 0.122% malachite green in 6 N H_2SO_4 and 0.2 ml of 0.11% Tween 20 were mixed to form the malachite green solution. 800 µl of sample was mixed with 200 µl of the malachite green solution. The experiment was repeated for different ADP

concentrations (0.1, 0.15, 0.2, 0.25 and 0.3 mM). Batch kinetics studies of PET-COOH-NTPDase were performed by exposing 2 cm^2 of PET-COOH-NTPDase to 1.5 ml of ADP in the presence of 0.1 M Tris buffer (pH = 7.4) and 5 mM $CaCl_2$ at 37ºC. The rate of Pi release was measured using the malachite green assay (Baykov *et al.* 1988). The experiment was repeated for different ADP concentrations (0.1, 0.15, 0.2, and 0.25 mM).

4.3 Results and Discussion

4.3.1 Free NTPDase kinetics

Free NTPDase kinetics were studied to understand the mechanism of enzyme reaction and to compare with immobilized NTPDase. Figure 4.1 shows the dimensionless formation of Pi (Pi formed/Initial [ADP]) in the free NTPDase studies.

As observed, the amount of Pi formed was very rapid at the beginning and reached a plateau after a short time. Furthermore, the results show that ADP didn't convert to AMP and Pi completely. The conversion of ADP ranged from about 60% to 80%. One of the possible reasons for such an observation could be the loss of NTPDase activity when it is in solution, leading to complete loss in activity after a given time. To test this hypothesis, NTPDase was stored for a given time in a solution composed of 0.1 M Tris buffer (pH = 7.4) and 5 mM $CaCl_2$. Afterwards, the activity of NTPDase was assessed by adding ADP to the solution. The results are shown in Figure 4.2.

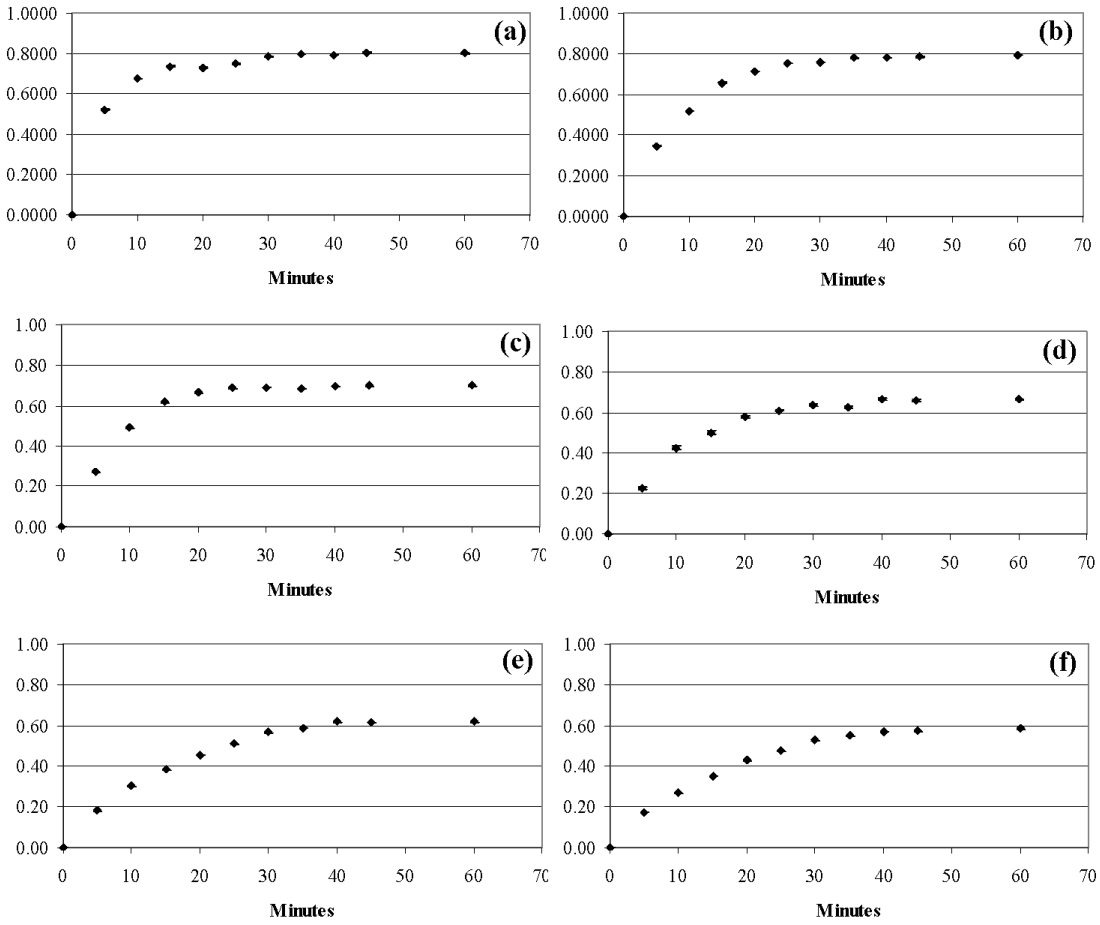


Figure 4.1. Dimensionless formation (Pi formed/Initial [ADP]) from free NTPDase with (a) Initial [ADP] = 0.05 mM, (b) Initial [ADP] = 0.1 mM, (c) Initial [ADP] = 0.15 mM, (d) Initial [ADP] = 0.2 mM, (e) Initial [ADP] = 0.25 mM and (f) Initial [ADP] = 0.3 mM.

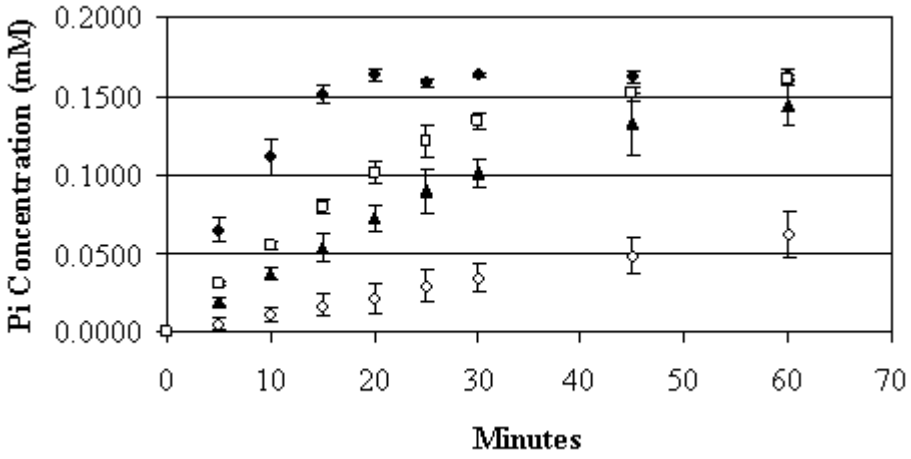


Figure 4.2. Effect of storing free NTPDase in 0.1 M Tris buffer at 37ºC for 0 minutes (♦), 60 minutes (□), 120 minutes (▲) and 240 minutes (◊).

As seen in these results, it is evident that NTPDase loses activity when stored in solution for long hours. However, the stability of PET-COOH-NTPDase (discussed in Chapter 3) showed that the polymer didn't lose any NTPDase activity even when it was stored in solution at 37ºC for 200 minutes (Figure 3.9). These results indicate that immobilization of NTPDase on carboxylated PET improves the temperature stability of NTPDase when stored in a solution. This increased stability of NTPDase activity due to immobilization would potentially enhance the haemocompatibility for long-term biomedical applications.

Due to the loss of NTPDase stability during free NTPDase kinetics, it was difficult to fit the Pi versus time curve for each experimental condition to the Michaelis-Menten equation. Therefore, the initial rates of Pi formation for different initial ADP concentrations were calculated. Using these initial rates and ADP concentrations, a Lineweaver-Burke plot was made (Figure 4.3). Using the slope and the intercept from the Lineweaver-Burke plot, the Michaelis-Menten constants v_{max} = 0.0118 mM/min (95% confidence range is 0.0105 to 0.0133) and K_M = 0.0643 mM (95% confidence range is 0.060 to 0.068) were calculated. As shown, the linear fit (R^2 = 0.984) agrees very well with the linear expectation of a Lineweaver-Burke plot. It is important to note that v_{max} includes not only the catalytic rate constant associated with NTPDase, but also the amount of enzyme in solution.

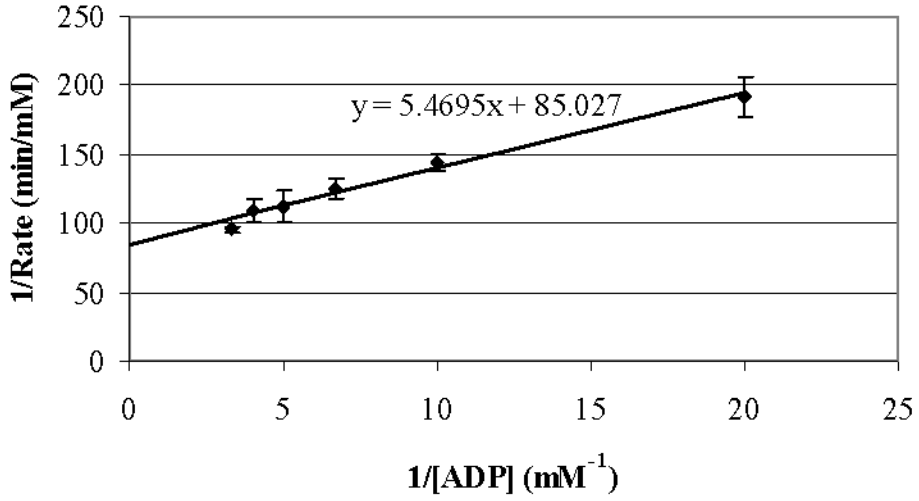


Figure 4.3. Lineweaver-Burke plot using initial rates from free NTPDase kinetics with different initial [ADP]. Using the slope and intercept the Michaelis-Menten constants were calculated as v_{max} = 0.0118 mM/min and K_M = 0.0643 mM.

4.3.2 Immobilized NTPDase kinetics (PET-NH_2-NTPDase)

For understanding the effect of immobilization on NTPDase kinetics, PET-NH_2-NTPDase polymers were analyzed. These kinetic studies and the analysis discussed in Chapter 3 regarding aminolyzed PET and PET-NH_2-NTPase were performed concurrently. The results obtained for the rate of Pi formation for different initial ADP concentrations are shown in Figures 4.4a, 4.5a, 4.6a, 4.7a and 4.8a. The first observation from the results was that the error bars for the concentrations were relatively big. The possible reasons could include the instability of imine bonds on PET-NH_2-NTPDase, loss of the activity of NTPDase that was detached from PET-NH_2-NTPDase, and the inconsistent development of cracks in the polymer (leading to varying amounts of NTPDase that could attach). Also, in the 50-ml reactor, where 5 cm × 1 cm polymer was cut into five pieces, it was observed that these pieces stuck to each other or to the wall of the reactor in some trials. This could make the experimental conditions different for the different trials, which could have also resulted in the big error bars.

Once the Pi concentrations over time were measured, ADP concentrations were calculated based on stoichiometry. These ADP concentrations were fit to a polynomial in terms of time. This equation for ADP concentration as a function of time was used to estimate the rate of change of ADP concentration. ADP concentrations and the corresponding rates of ADP consumption were fit to a Michaelis-Menten model using SigmaPlot (Figures 4.4b, 4.5b, 4.6b, 4.7b and 4.8b).

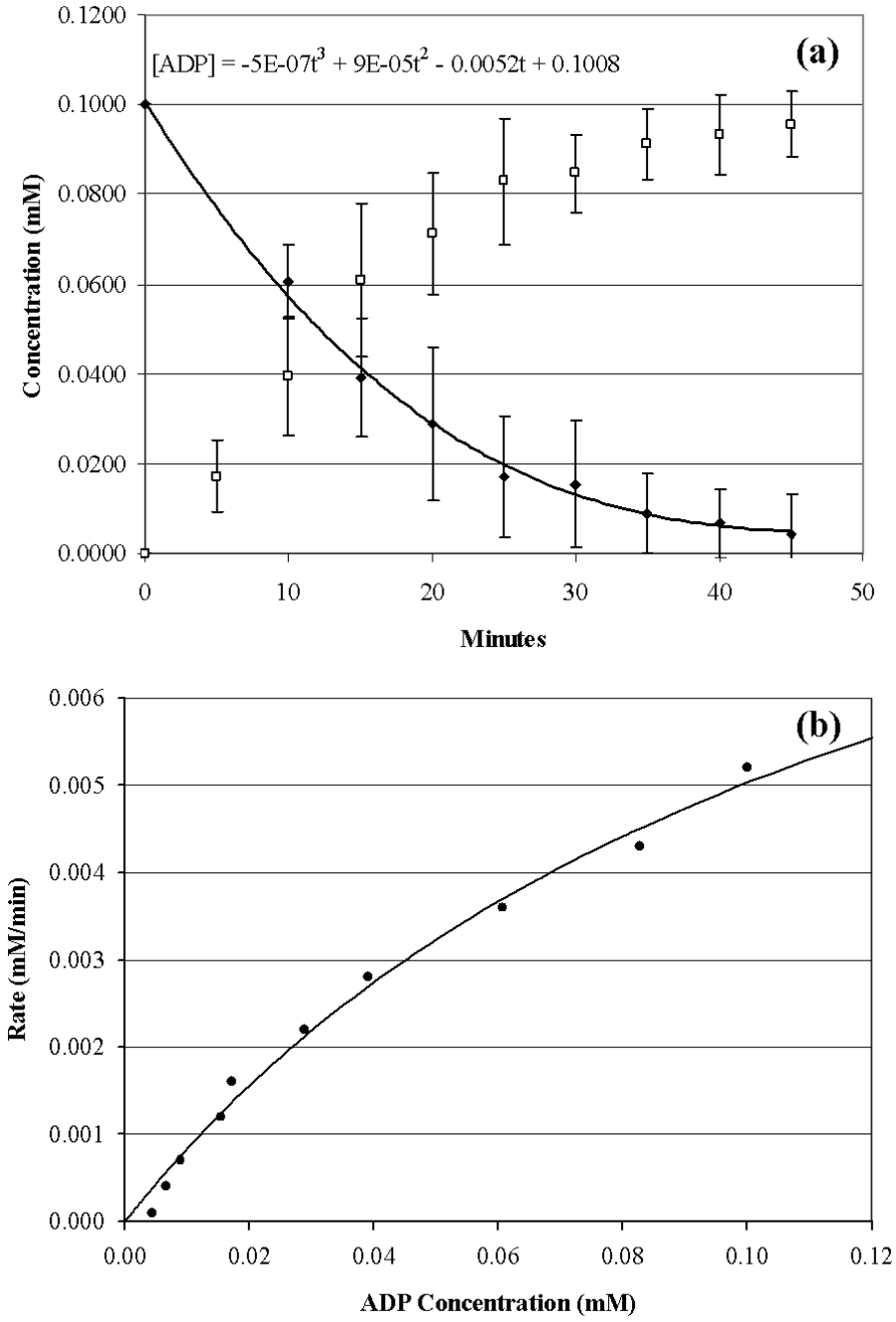


Figure 4.4. (a) NTPDase reaction kinetics for PET-NH_2-NTPDase using [ADP] = 0.1 mM. Pi (□) and ADP (♦) concentrations are shown. The ADP concentration was fitted to a polynomial for estimating the derivative, (b) Rate of ADP conversion using PET-NH_2-NTPDase for initial [ADP] = 0.1 mM. Solid line is the Michaelis-Menten model with v_{max} = 0.01131 mM/min and K_M = 0.1247 mM

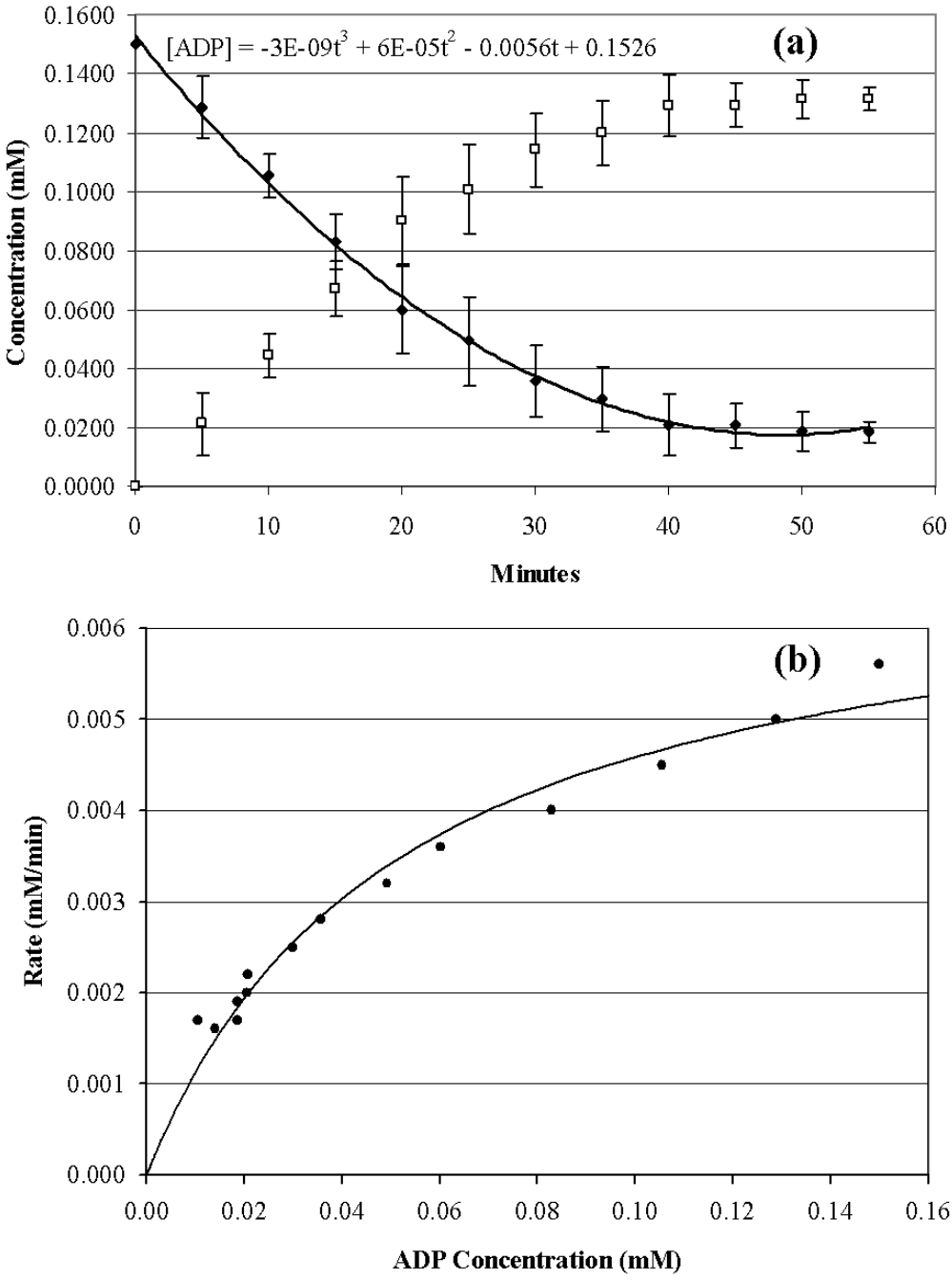


Figure 4.5. (a) NTPDase reaction kinetics for PET-NH_2-NTPDase using [ADP] = 0.15 mM. Pi (□) and ADP (♦) concentrations are shown. ADP concentration was fitted to a polynomial for estimating the derivative, (b) Rate of ADP conversion using PET-NH_2-NTPDase for initial [ADP] = 0.15 mM. Solid line is the Michaelis-Menten model with v_{max} = 0.006949 mM/min and K_M = 0.05151 mM

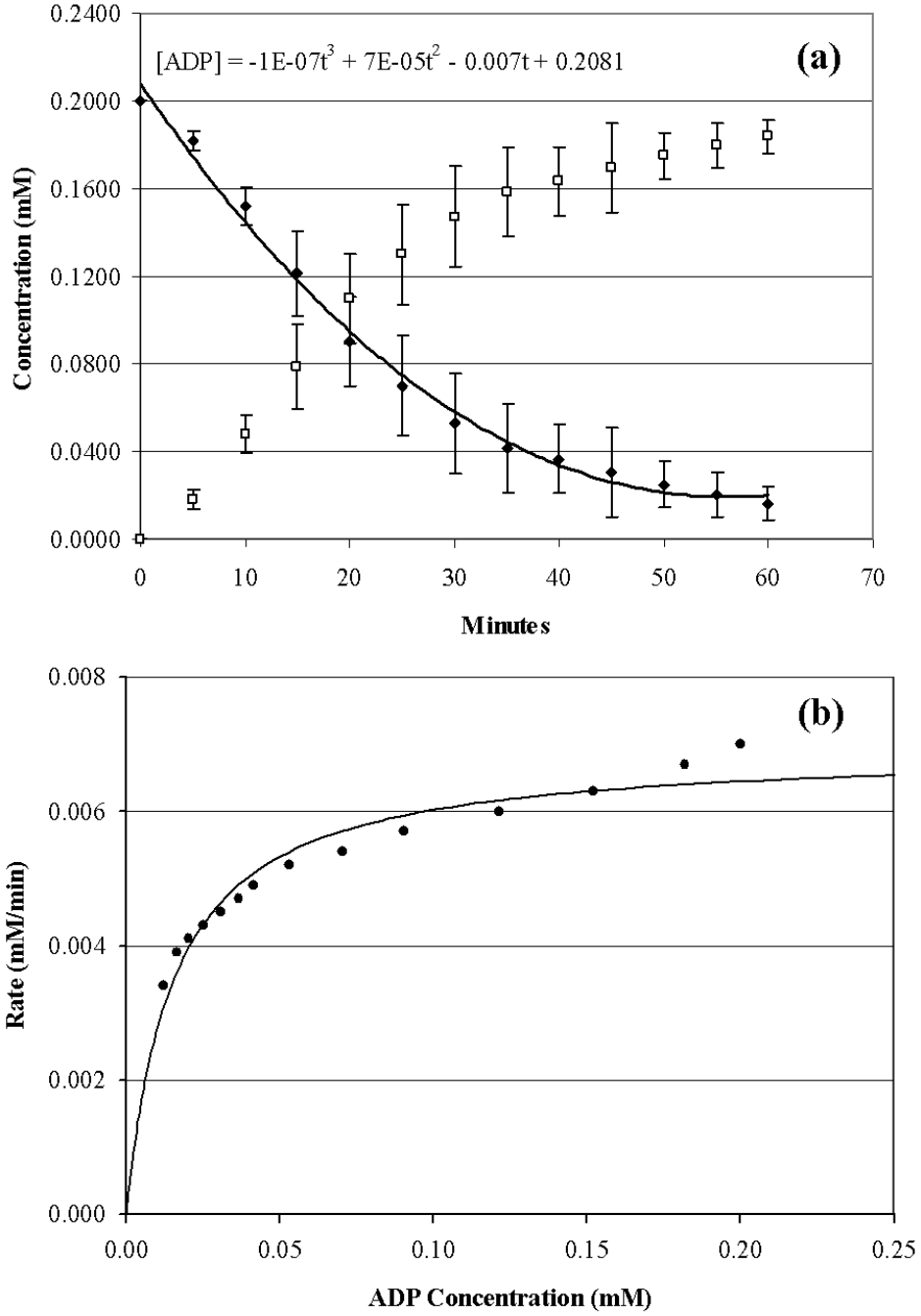


Figure 4.6. (a) NTPDase reaction kinetics for PET-NH_2-NTPDase using [ADP] = 0.2 mM. Pi (□) and ADP (♦) concentrations are shown. ADP concentration was fitted to a polynomial for estimating the derivative, (b) Rate of ADP conversion using PET-NH_2-NTPDase for initial [ADP] = 0.2 mM. Solid line is the Michaelis-Menten model with v_{max} = 0.06934 mM/min and K_M = 0.01509 mM

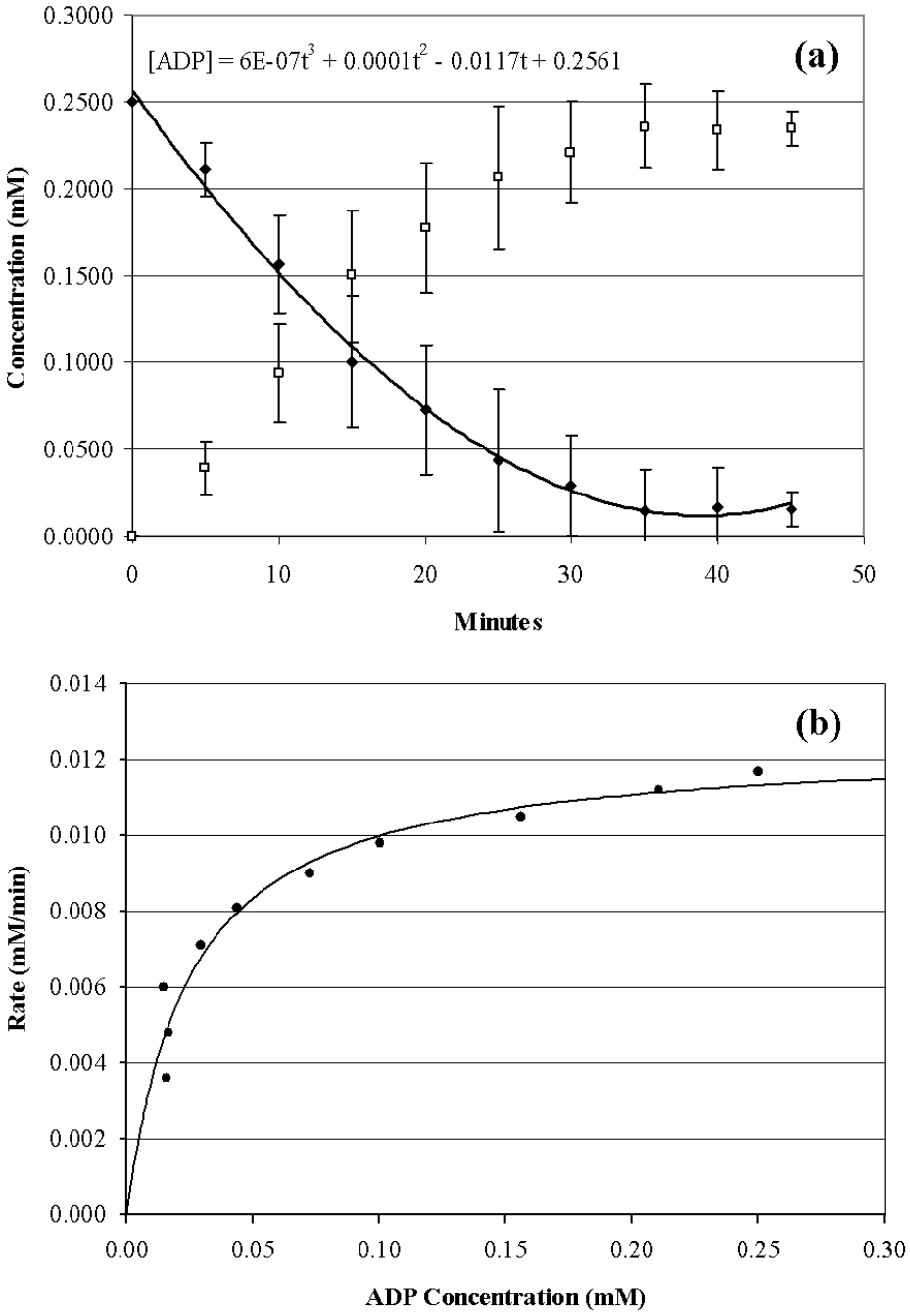


Figure 4.7. (a) NTPDase reaction kinetics for PET-NH_2-NTPDase using [ADP] = 0.25 mM. Pi (□) and ADP (♦) concentrations are shown. ADP concentration was fitted to a polynomial for estimating the derivative, (b) Rate of ADP conversion using PET-NH_2-NTPDase for initial [ADP] = 0.25 mM. Solid line is the Michaelis-Menten model with v_{max} = 0.01242 mM/min and K_M = 0.02425 mM

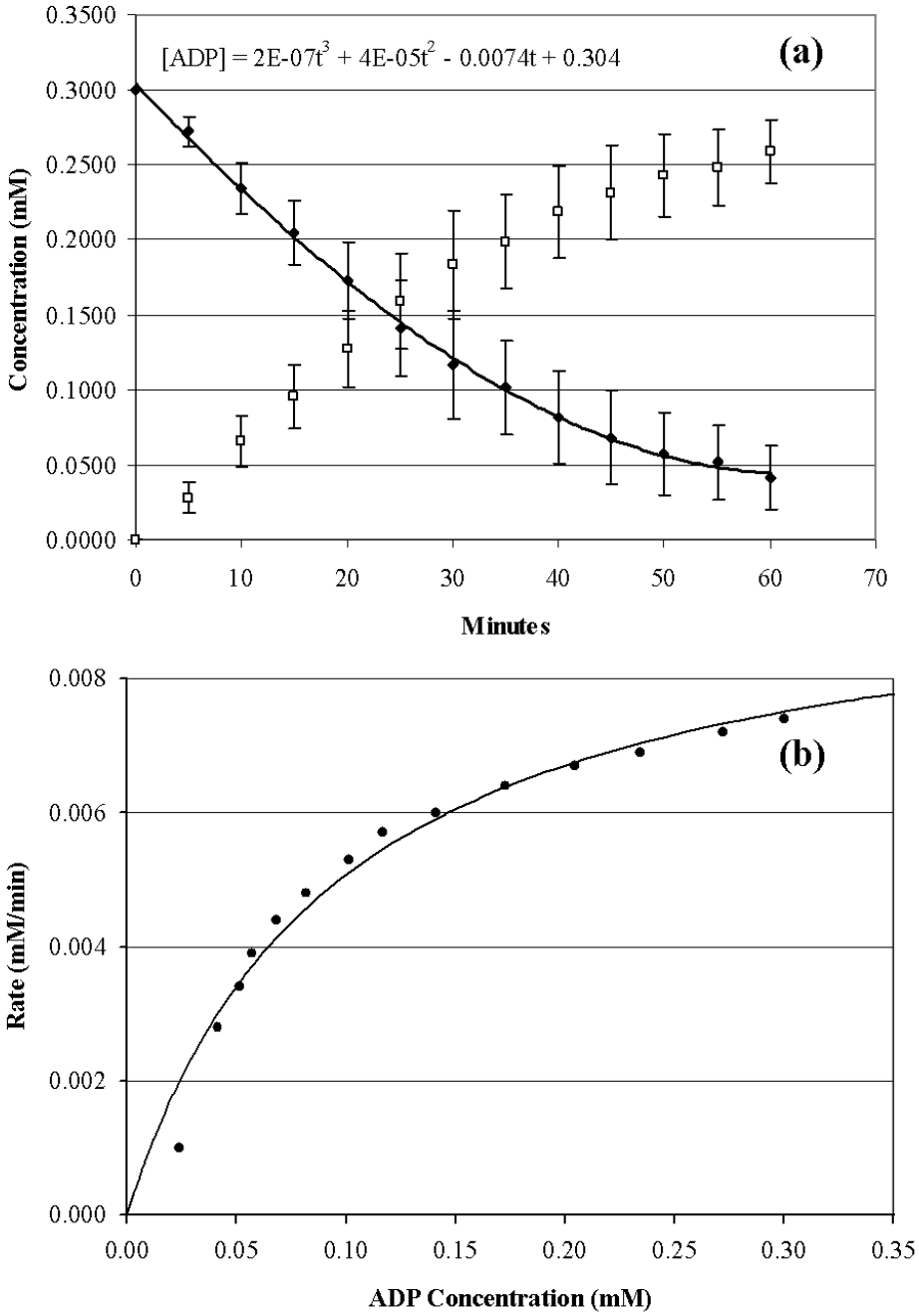


Figure 4.8. (a) NTPDase reaction kinetics for PET-NH_2-NTPDase using [ADP] = 0.3 mM. Pi (□) and ADP (♦) concentrations are shown. ADP concentration was fitted to a polynomial for estimating the derivative, (b) Rate of ADP conversion using PET-NH_2-NTPDase for initial [ADP] = 0.3 mM. Solid line is the Michaelis-Menten model with v_{max} = 0.009873 mM/min and K_M = 0.09461 mM

From these plots, it was observed that the rates approached saturated kinetics as ADP concentrations increased—this is consistent with Michaelis-Menten kinetics. For example, at initial [ADP] = 0.1 mM, the rate of reaction never approached a saturation limit. Although most of the data obtained seemed to fit Michaelis-Menten kinetics, it was observed that the Michaelis-Menten constants were inconsistent for different initial ADP concentrations. It should be noted that some of the data was extrapolated to obtain the constants, which is problematic.

As PET-NH_2-NTPDase was stored at -20ºC (recommended storage condition for free NTPDase) for up to 7 days for some of the trials, it was important to verify if PET-NH_2-NTPDase lost any activity during storage. For this reason, PET-NH_2-NTPDase was stored at -20ºC for 4 weeks and then tested for activity using 0.2 mM ADP. The results obtained, shown in Figure 4.9, indicate that there was no measureable loss of NTPDase activity due to storage at -20ºC. As storing the polymer didn't affect the activity, the reasons for different Michaelis-Menten constants for different initial ADP concentrations could include instability of PET-NH_2-NTPDase immobilization, loss of NTPDase activity after NTPDase was detached from the polymer and the uncertainties associated with the 50-ml experimental system.

With multiple factors regulating the kinetics in these experiments, rigorous analysis would be required to understand the reason behind different Michaelis-Menten constants. As aminolyzed PET didn't have desired surface and bulk properties and PET-NH_2-NTPDase was not a stable immobilization, further analysis was not performed.

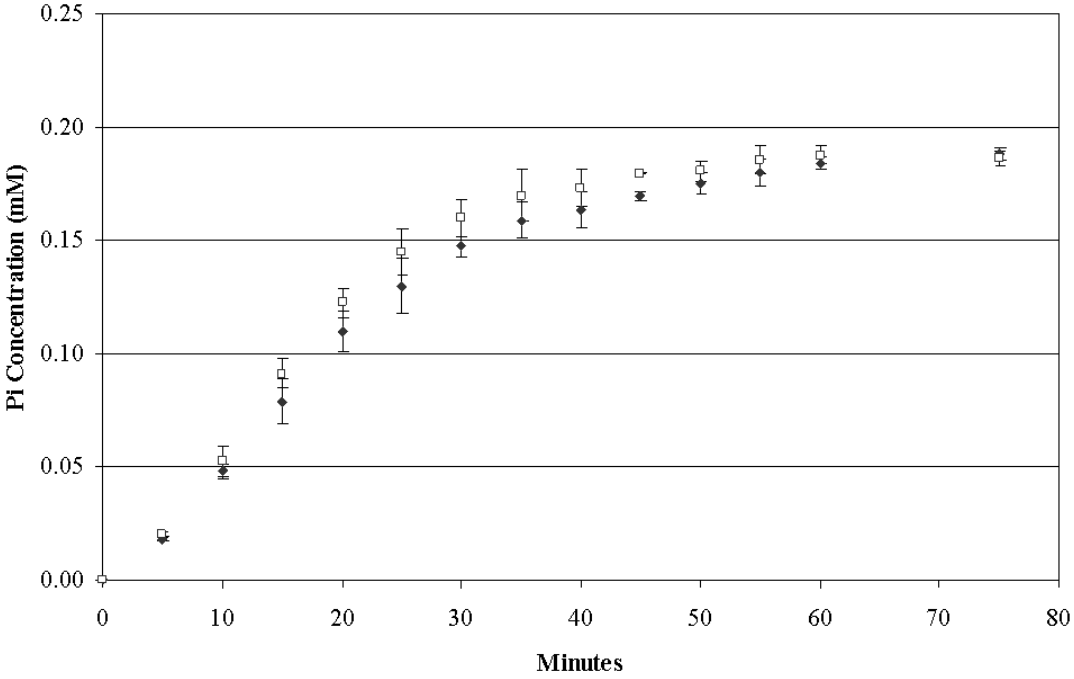


Figure 4.9. Effect of storing PET-NH_2-NTPDase at -20ºC for 4 weeks. Pi formation for freshly prepared PET-NH_2-NTPDase (♦) and PET-NH_2-NTPDase stored at -20ºC for 4 weeks (□), with 0.2 mM ADP, are shown.

4.3.3 Immobilized NTPDase kinetics (PET-COOH-NTPDase)

To overcome the limitations seen in PET-NH_2-NTPDase, immobilized NTPDase kinetics was studied using PET-COOH-NTPDase. As results discussed in Chapter 3 showed that 50-ml experimental could cause mass transfer limitations, PET-COOH-NTPDase kinetics were performed using small volume reactors. Pi concentrations at different times were measured using the malachite green assay. With the Pi concentrations at different times, the corresponding ADP concentrations were calculated using stoichiometry. This ADP concentration was plotted and fitted to a polynomial. The derivative of this polynomial was used to estimate the rate of ADP conversion for any given ADP concentration. Figures 4.10a, 4.11a, 4.12a and 4.13a show the Pi concentration, ADP concentration and the polynomial used to fit ADP concentration in terms of time. The results show that the error bars are very small, although the error could produce significant problems with estimating kinetic constants (see below).

Using the polynomial for ADP concentration, the rates of ADP conversion were calculated and plotted against the corresponding ADP concentration. This plot was fit to a Michaelis-Menten model using SigmaPlot as shown in Figures 4.10b, 4.11b, 4.12b and 4.13b. The results showed that most of the data obtained from the experiments were around the saturation condition, which makes it difficult to obtain good Michaelis-Menten kinetic values. Even for low concentrations of ADP, such as [ADP] = 0.1 mM, the data suggests saturation.

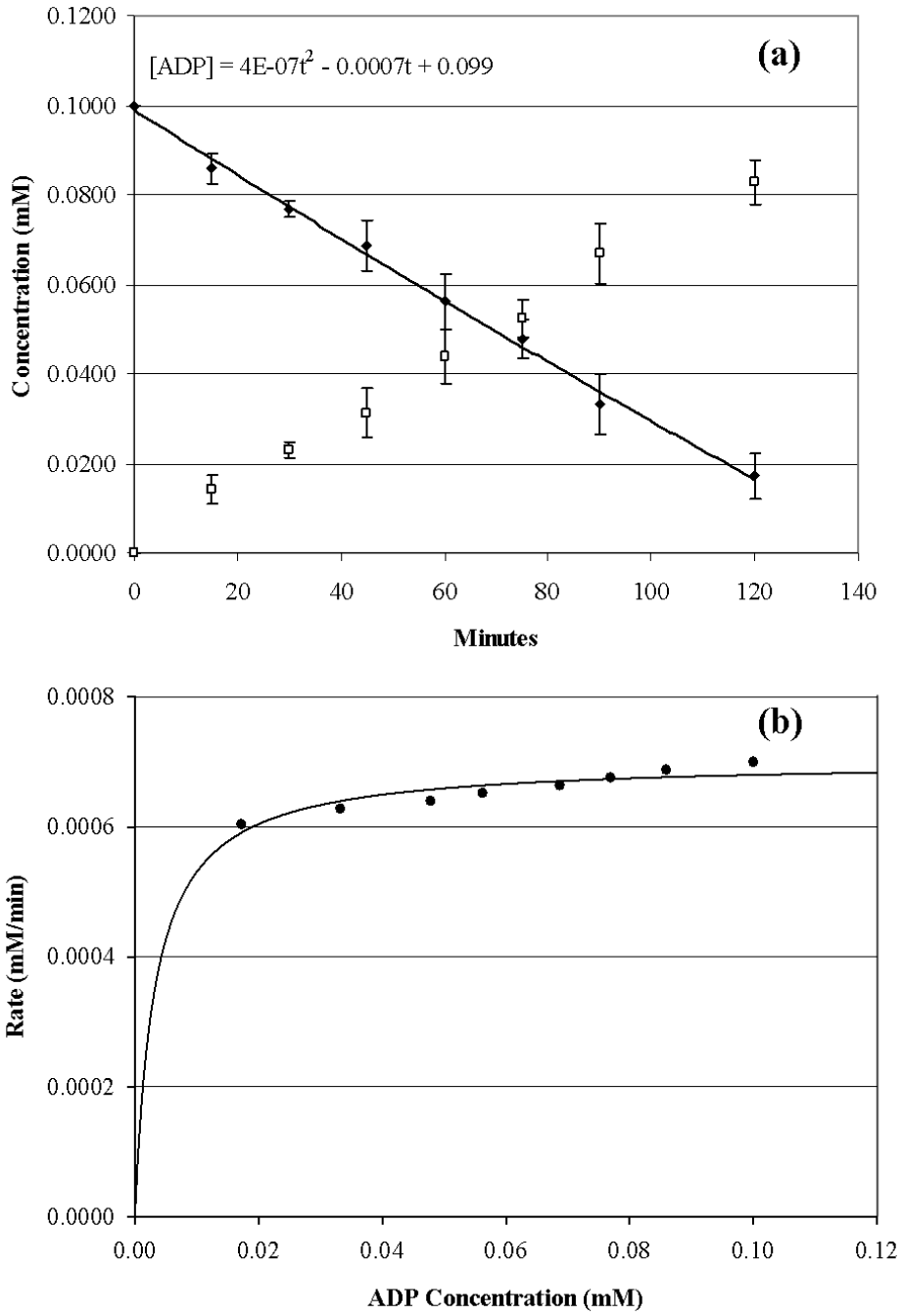
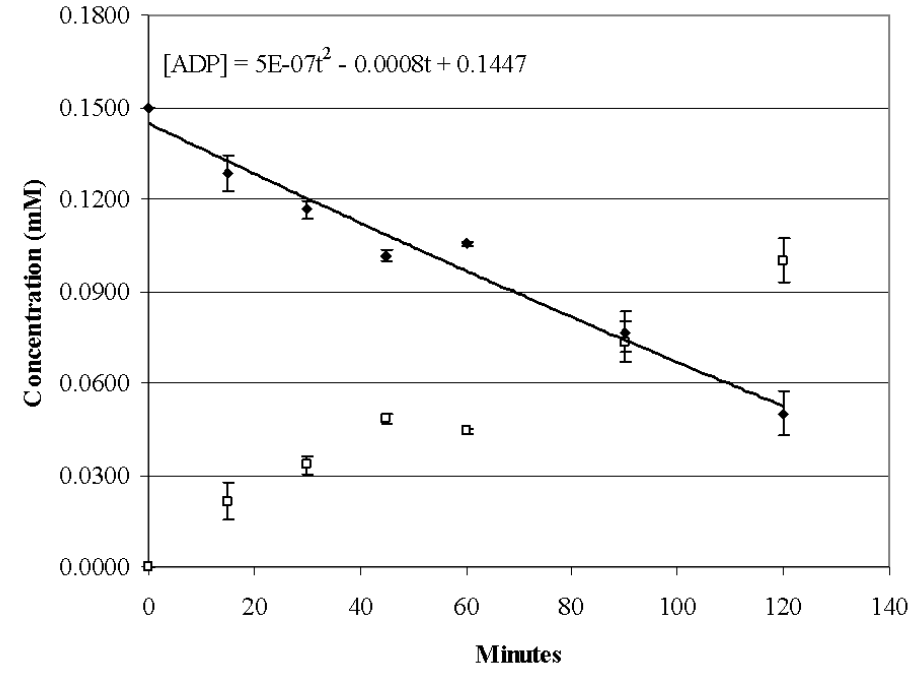


Figure 4.10. (a) NTPDase reaction kinetics for PET-COOH-NTPDase using [ADP] = 0.1 mM. Pi (□) and ADP (♦) concentrations are shown. ADP concentration was fitted to a polynomial for estimating the derivative, (b) Rate of ADP conversion using PET-COOH-NTPDase for initial [ADP] = 0.1 mM. Solid line is the Michaelis-Menten model with v_{max} = 0.000702 mM/min and K_M = 0.003225 mM



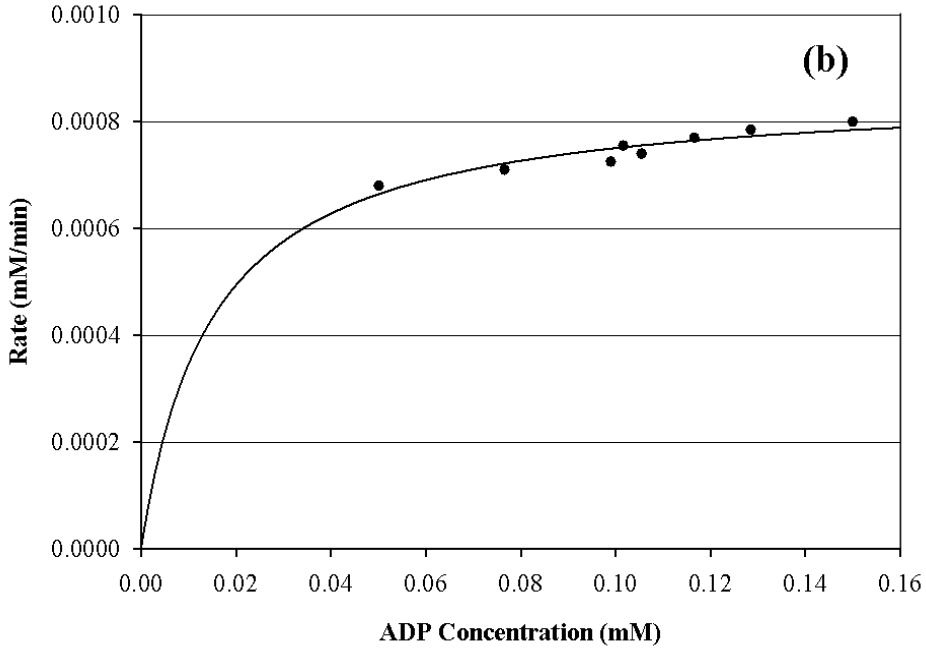


Figure 4.11. (a) NTPDase reaction kinetics for PET-COOH-NTPDase using [ADP] = 0.15 mM. Pi (□) and ADP (♦) concentrations are shown. ADP concentration was fitted to a polynomial for estimating the derivative, (b) Rate of ADP conversion using PET-COOH-NTPDase for initial [ADP] = 0.15 mM. Solid line is the Michaelis-Menten model with v_{max} = 0.000863 mM/min and K_M = 0.01494 mM

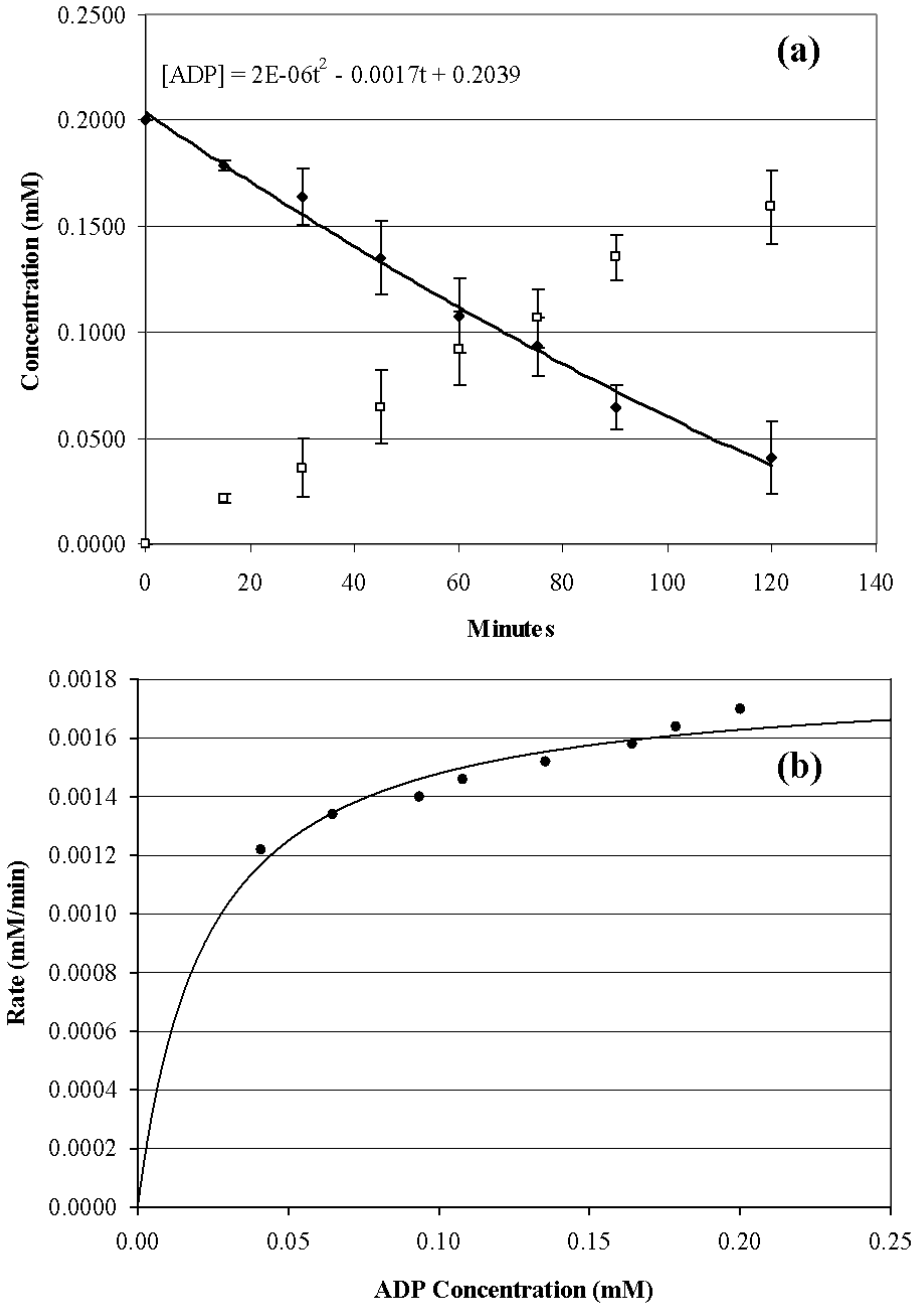


Figure 4.12. (a) NTPDase reaction kinetics for PET-COOH-NTPDase using [ADP] = 0.2 mM. Pi (□) and ADP (♦) concentrations are shown. ADP concentration was fitted to a polynomial for estimating the derivative, (b) Rate of ADP conversion using PET-COOH-NTPDase for initial [ADP] = 0.2 mM. Solid line is the Michaelis-Menten model with v_{max} = 0.00181 mM/min and K_M = 0.02246 mM

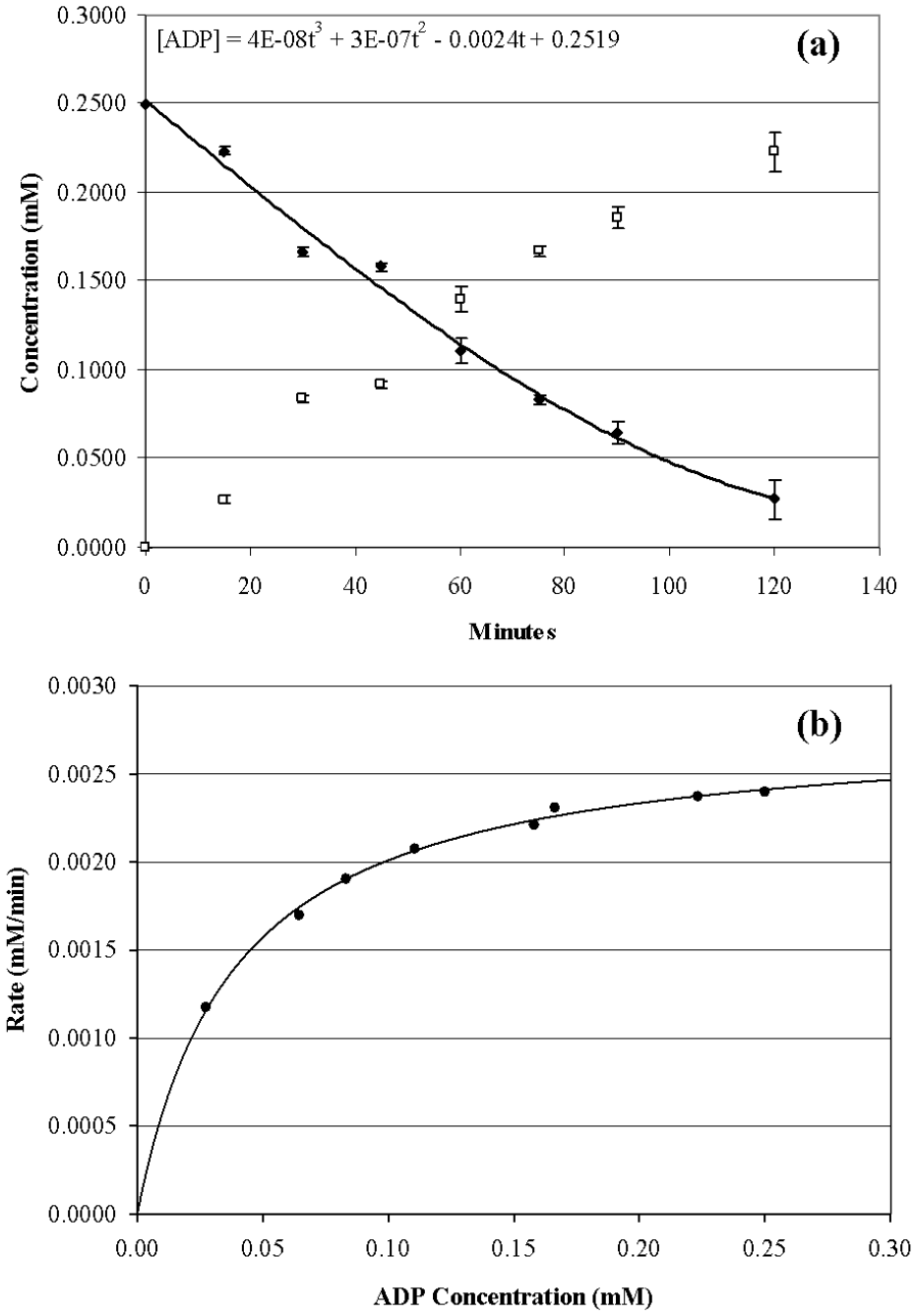


Figure 4.13. (a) NTPDase reaction kinetics for PET-COOH-NTPDase using [ADP] = 0.25 mM. Pi (□) and ADP (♦) concentrations are shown. ADP concentration was fitted to a polynomial for estimating the derivative, (b) Rate of ADP conversion using PET-COOH-NTPDase for initial [ADP] = 0.25 mM. Solid line is the Michaelis-Menten model with v_{max} = 0.00278 mM/min and K_M = 0.03848 mM

As noted, different initial ADP concentration experiments resulted in different v_{max} and K_M values. It was also observed that both v_{max} and K_M calculated using SigmaPlot increased with an increase in the initial ADP concentrations (Table 4.1 and Figure 4.14). It's possible for v_{max} to vary among experiments since v_{max} is proportional to the amount of NTPDase on a polymer but K_M should be consistent. However, the calculation of K_M value is very sensitive to the rate of Pi formation at low substrate concentrations. Thus, accurate measurement of these rates is required for precise calculation of K_M values.

Data in Figures 4.10a, 4.11a and 4.12a show that the error bars are bigger for Pi concentrations measured during the later stages of the experiment. For instance, ADP concentrations calculated from the measured Pi concentrations at 75, 90, and 120 minutes for the 0.1 mM study are 0.0478 ± 0.0042 mM, 0.0332 ± 0.0067 and 0.0172 ± 0.0050 mM, respectively. As one can see, the measurement of Pi concentration, and therefore the corresponding ADP concentration, is not sensitive enough to measure smaller changes. This limitation has resulted in relatively larger error bars for Pi concentrations measured towards the final stages of the experiment. However, for calculating the rate of Pi formation at lower ADP concentrations, accurate measurement of Pi concentration is required. Since the data obtained shows variability at lower ADP concentrations, it is difficult to identify the actual rates at these concentrations. If the rate of Pi formation is lower, which is a distinct possibility, at lower ADP concentrations, K_M values would be higher from the Michaelis-Menten curves. Therefore, further studies using protocols that are sensitive towards small changes in Pi concentrations are required for accurately measuring the rates for lower ADP concentrations and, thereby, accurate K_M values.

Table 4.1. v_{max} and K_M calculated using Michaelis-Menten model for different initial ADP concentrations

Initial [ADP], mM	v_{max}, mM/min	K_M, mM
0.1	0.000702	0.003225
0.15	0.000863	0.01494
0.2	0.001811	0.02246
0.25	0.002783	0.03848

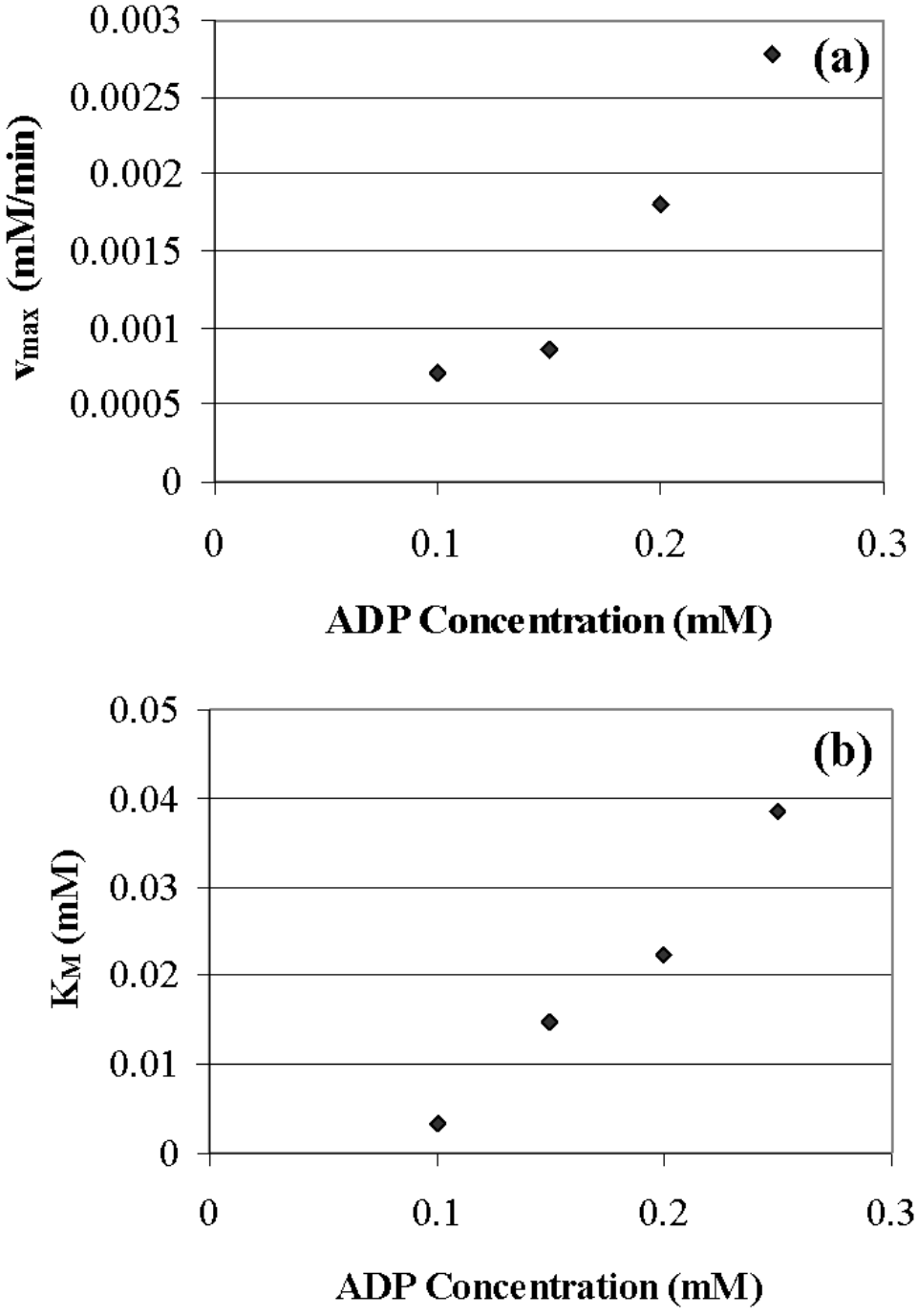


Figure 4.14. (a) v_{max} vs. initial ADP concentrations, (b) K_M vs. initial ADP concentrations.

Although error bars observed for Pi concentrations at very low ADP concentrations are bigger for the initial ADP studies less than 0.2 mM, it is important to note that the error bars were the smallest for the study with an initial ADP of 0.25 mM. These smaller error bars will give more accurate analysis of the constants. The data for the 0.25 mM study would have far less error in determining the Michaelis-Menten constants since the error bars are extremely small—perhaps a result of assessing Pi changes over larger concentration ranges. It's interesting that the K_M for the 0.25 mM immobilized study (0.038 mM) is the closest to the K_M for the free NTPDase studies (0.064 mM). The amount of error, although initially appearing small, may be a large reason as to the inconsistent kinetic analysis.

By definition, v_{max} is the product of the enzyme rate constant and the concentration of the enzyme. Therefore, comparing the v_{max} values obtained for free NTPDase studies and the PET-COOH-NTPDase study performed with 0.25 mM initial ADP could provide information regarding NTPDase coverage on the PET-COOH-NTPDase. Assuming one NTPDase molecule (MW 47,000) is spherical (with a radius of 2.7 nm-see (Fournier 1999) for estimating radius), the maximum theoretical coverage of NTPDase by forming a tightly packed monolayer was calculated to be 0.0073 nmoles/cm^2. Thus, in the 1.5 ml experiment, the concentration of NTPDase would be 9.7 nM. For the free enzyme study, the concentration was approximately 1.3 nM. Assuming the enzyme rate constant doesn't change due to immobilization, the ratio of v_{max} for free NTPDase to v_{max} of PET-COOH-NTPDase with a maximum theoretical coverage would be equivalent to the ratio of the free NTPDase concentration to the NTPDase concentration in the PET-COOH-NTPDase study. From the above reported

values, this NTPDase concentration ratio is 0.13 if NTPDase had complete coverage on the surface. Thus, the v_{max} ratio would be 0.13. From the experimental data, the ratio of v_{max} for free NTPDase to v_{max} calculated for PET-COOH-NTPDase studies using 0.25 mM initial ADP is 4.2. Using this v_{max} ratio, the equivalent NTPDase ratio of 0.13 would suggest that the NTPDase concentration in the PET-COOH-NTPDase study is 0.13 nM. This concentration would equate to 1.3% of the maximum theoretical coverage. Although this seems reasonable, further work would need to be performed to assess the coverage.

As noted above, it was difficult to accurately obtain the Michaelis-Menten constants for PET-COOH-NTPDase, although the 0.25 mM ADP study gave values closest to the free NTPDase studies. As seen for initial [ADP] = 0.25 mM, the Michaelis-Menten equation provides a very good fit. Based on these results, it is evident that the reactions that were carried out in 1.5-ml reactors do not appear to be mass transfer limited since the data fits a reaction limited equation (i.e. Michaelis-Menton). One of the possible scenarios describing differences in the rate of Pi formation in the 50-ml and 1.5-ml reactors, as discussed in Chapter 3, was different limiting conditions for the two reactors. As seen in these results, it is reasonable to expect that reactions occurring in 1.5-ml reactors are reaction kinetic limited.

Another interesting observation was that experiments using $[ADP]_0$ = 0.25 mM were performed first, followed by $[ADP]_0$ = 0.2 mM, $[ADP]_0$ = 0.15 mM and $[ADP]_0$ = 0.1 mM. Therefore, experiments with $[ADP]_0$ = 0.25 mM used fresh PET-COOH-NTPDase and $[ADP]_0$ = 0.1 mM used PET-COOH-NTPDase that had been stored the longest (approximately 12 hours). Again, as previously mentioned, the first study gave

the K_M value closest to the free NTPDase K_M value. Free NTPDase is recommended to be stored at -20ºC. Also, NTPDase activity was not affected when PET-NH_2-NTPDase was stored at -20ºC for 4 weeks. Hence, PET-COOH-NTPDase was stored at -20ºC, when not used. However, PET-COOH-NTPDase showed improved temperature stability in solution at 37ºC compared to free NTPDase. Therefore, it is possible that the effect of temperature is different for PET-COOH-NTPDase compared to free NTPDase and PET-NH_2-NTPDase. Further studies to verify if storing PET-COOH-NTPDase at -20ºC has an effect on NTPDase activity could provide additional insight into explaining the results.

4.4 Conclusions

Free NTPDase kinetics were studied and modeled using the Michaelis-Menten equation. The results obtained show that free NTPDase lost activity, when it was stored in a solution at 37ºC. However, from the results discussed in Chapter 3, PET-COOH-NTPDase didn't lose any activity when it was stored in solution at 37ºC. These results suggest that immobilizing NTPDase provides improved temperature stability at 37ºC. This could probably improve the potential of the polymer in long-term biomedical applications. Immobilized NTPDase kinetics were studied using PET-NH_2-NTPDase and PET-COOH-NTPDase. PET-NH_2-NTPDase kinetics were inconclusive as NTPDase immobilization was not stable. PET-COOH-NTPDase kinetics were also analyzed. Results obtained indicate that PET-COOH-NTPDase kinetic studies performed in the 1.5-ml reactors appear to be kinetic limited and follow Michaelis-Menten kinetics. However, Michaelis-Menten constants for these reactions couldn't be calculated with confidence

due to the limitations in the sensitivity of Pi measurements. Also, more studies are required to understand the effect of storing PET-COOH-NTPDase at -20ºC. Although the immobilized NTPDase kinetics couldn't be modeled effectively, it is important to recognize that all PET-COOH-NTPDase polymers showed significant NTPDase activity. In addition to that, the results also indicate an increased stability of NTPDase activity in solution at 37ºC, due to immobilization.

CHAPTER 5

IN VITRO STUDIES OF NTPDase-MODIFIED POLYETHYLENE TEREPHTHALATE TO VERIFY IMPROVED HAEMOCOMPATIBILITY

Haemocompatibility is the compatibility of a biomaterial when it comes in contact with blood. It is an important criterion in developing a biomaterial used in blood-contacting systems. The lack of haemocompatibility is a serious problem that limits the functionality of many biomaterials. Based on the Manufacturer and User Facility Device Experience (MAUDE) Database, at least 1510 cases of thrombosis in implants and other biomedical devices, of which 299 resulted in deaths, have been reported to the FDA in 2008. These numbers have increased drastically from 2003, when 593 cases of thrombosis, of which 113 resulted in death, were reported. Such thrombosis events are occurring even with aggressive use of antithrombotic drugs, which can cause bleeding complications (van Oeveren 2005). The new types of catheters and stents that are being used today have actually caused an increase in the thrombotic events that are reported (van Oeveren 2005). Improving the haemocompatibility of a biomaterial will significantly improve the current situation. This will save lives and improve the quality of life for people living with implants. While developing a material for blood-contacting devices, testing the haemocompatibility is crucial. Understanding the blood-material interactions gives valuable information required in designing a biomaterial.

With surface and bulk characterization proving that PET-COOH-NTPDase has desirable properties, PET-COOH-NTPDase has the potential to be a valuable biomaterial. The polymer can have vast applications in blood-contacting systems, if the haemocompatibility is improved due to ADP scavenging by NTPDase. For testing this hypothesis, the haemocompatibility of PET-COOH-NTPDase was analyzed using *in vitro* studies. In this chapter, protein adsorption on unmodified, carboxylated and NTPDase-modified PET was analyzed. Platelet adhesion on unmodified, carboxylated and NTPDase-modified PET when exposed to platelet rich plasma was also studied. This chapter also discusses thrombus formation when unmodified, carboxylated and NTPDase-modified PET were exposed to whole blood. Results obtained from these batch studies should provide greater understanding on the haemocompatibility of PET-COOH-NTPDase.

5.1 Introduction

The significance of haemocompatibility in developing a blood-contacting material can't be overstated. The lack of haemocompatibility causes serious complications in patients that could even lead to death. The use of biomedical devices, such as hemodialyzers, vascular grafts, heart valve prostheses, artificial heart etc., which come in contact with blood, is growing rapidly. Hence, it is vital to understand the haemocompatibility of materials that are used in blood-contacting systems. Extensive testing of materials is required to understand blood compatibility. Many experimental procedures have been developed to evaluate the effect of a material on blood coagulation

and thrombus formation. For a material to be used in a commercial biomedical device, it has to go through a series of biocompatibility and haemocompatibility testing. The regulations for testing these biomaterials are provided by many organizations, including the American Society of Testing and Materials International (ASTM), International Standards Organization (ISO), the American Dental Association (ADA), the National Institutes of Health (NIH) and the Food and Drug Administration (FDA). The first phase of testing the haemocompatibility is *in vitro* studies. *In vitro* studies are performed to screen materials that could potentially be used in biomedical applications. The next step is using an *ex vivo* system to evaluate the haemocompatibility under a more *in vivo*-like setup. In the final stage involving lab-scale testing, *in vivo* experiments using animal models are performed. Ultimately, clinical trials are designed to evaluate the safety of the biomaterial when used in humans. Such clinical trials are performed only after extensive *in vitro* and *in vivo* animal model testing has been done. However, clinical trials are usually intended towards testing the biomedical device as a complete unit, instead of testing the biomaterials used in building the device.

5.1.1 *In vitro* systems

In vitro testing is the most common and fundamental approach used to evaluate the material's haemocompatibility. *In vitro* experiments are easier, faster, less expensive and safer compared to the *in vivo* studies. The experiments are performed in a more controlled environment. Hence, they provide a basic understanding of the material properties. There are many types of *in vitro* systems used for evaluating blood

compatibility. Protein adsorption studies and static studies, performed using platelet-rich plasma and human blood, are common *in vitro* experiments used for studying the potential of a biomaterial (Iwasaki *et al.* 1996; Li and Ruckenstein 2004; Joseph *et al.* 2008; Liu *et al.* 2008; Lin *et al.* 2009). Protein adsorption is important to analyze as it is the first event when the material comes in contact with blood. Further interactions of the blood with the material are mediated by a layer of protein (Wilson and Cooper 1986). Therefore, protein adsorption is known to have a great influence on the thrombogenic nature of the material. Currently many different techniques are employed in studying protein adsorption on a polymer surface. Protein adsorption on a polymer surface can be quantified using simple colorimetric assays (Ishihara *et al.* 1999b; Lin *et al.* 2004; Pan *et al.* 2007; Liu *et al.* 2008). Conformational changes in the adsorbed proteins have also been explored using an ATR-FTIR flow cell method (Ito *et al.* 1986; Sanada *et al.* 1986), circular dichroism (Mcmillin and Walton 1974; Norde and Favier 1992) and calorimetric analysis (Chiu *et al.* 1976).

Interactions between platelets and the material being investigated are studied by simple static systems. Platelet-rich plasma studies have been used extensively for this reason (Kang *et al.* 1997; Lee *et al.* 1998; Li and Ruckenstein 2004; Wu *et al.* 2007; Liu *et al.* 2008). In these experiments, the material comes in direct contact with platelet-rich plasma (PRP). Platelets that are activated by exposure to foreign surfaces trigger the intrinsic pathway of the blood coagulation cascade. This will result in platelet adhesion and aggregation on the material. Studying the PRP-material interaction can provide some valuable information about the haemocompatibility of the material. Platelet adhesion and aggregation on the surface can be analyzed using scanning electron microscope (Iwasaki

et al. 1996; Li and Ruckenstein 2004; Liu *et al.* 2008) or by measuring the platelet concentration using a lactate dehydrogenase assay (Tamada *et al.* 1995). Although PRP studies are useful for understanding the haemocompatibility of a material, thrombus formation has to be analyzed. For this purpose, whole blood studies are used (Kang *et al.* 1997; Lin *et al.* 2004; Liu *et al.* 2008; Lin *et al.* 2009). In the whole blood studies, blood is contacted with a test surface for a fixed period of time. After this, both the blood and the surface may be examined. The most common tests performed on the blood are a partial thromboplastin time test, a platelet factor-3 assay, a prothrombin time test and counting of the platelet. The test surface exposed to blood can also be examined after blood contact. The degree of thrombus formation is usually measured using light, phase-contrast or scanning electron microscopy (Wilson and Cooper 1986).

5.1.2 *Ex vivo* systems

In an *ex vivo* system, blood flows from the test animal via a shunt and through the test surface. The blood-material interactions in the test area are monitored. *Ex vivo* studies are usually performed to evaluate short-term interactions. Both arteriovenous and arterioarterial shunts may be used for performing *ex vivo* studies. Many configurations of *ex vivo* experiments are available. One basic experiment is the Dudley clotting time test (Dudley *et al.* 1976; Wilson and Cooper 1986). In the Dudley clotting time test, a tube is inserted into the vein of a test animal. The time taken for the blood to stop dripping from the end of the tubing is called the Dudley clotting time. The clotting times can be compared for different materials by changing the tubing properties.

5.1.3 *In vivo* systems

Systemic and physiological processes are complex and are difficult to be simulated *in vitro*. Therefore, *in vivo* animal testing is necessary prior to human clinical testing. *In vivo* haemocompatibility tests are usually designed to simulate the geometry, flow dynamics and contact conditions of the material when used in its application. Although using *in vivo* animal testing is convenient, it is important to recognize that different species have different blood reactivity. This has to be considered while analyzing the data from *in vivo* animal models. *In vivo* haemocompatibility testing using animals is also complicated by the lack of adequate test materials such as antibodies for immunoassays. Also, the regulations of the governing bodies might restrict the use of some species in animal testing. For example, an arteriovenous shunt model in baboons shows results that are usually consistent with humans. However, non-human primates, which have blood reactivity very close to humans, can't be used as test animals in Europe.

5.1.4 Clinical trials

Clinical trials are performed after extensive research. Although clinical trials give information regarding the biomaterial, they are usually designed to test the effectiveness of the biomedical device that is built from the material. Usually in the clinical trials for

implants, the condition of the patient is compared before and after the implant and the implant is also compared with similar implants using other materials.

The first step in evaluating the haemocompatibility of a biomaterial is performing *in vitro* studies. For this reason, this research concentrates on understanding blood-material interactions *in vitro*. As discussed earlier, protein adsorption can play a vital role in the haemocompatibility of the biomaterial. In addition, the most common measures of thrombogenicity are platelet adhesion and thrombus formation. Hence this chapter explores protein adsorption, platelet adhesion and thrombus formation. Protein adsorption onto PET-COOH and PET-COOH-NTPDase were compared to the protein adsorption on unmodified PET. Platelet adhesion and thrombus formation on the modified PET surfaces were also analyzed using PRP and whole blood studies performed under static conditions.

5.2 Materials and methods

Polyethylene terephthalate (PET) (thickness = 0.2 mm) was supplied by DuPont (Hopewell, VA). Bromoacetic acid, 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), glucose, bovine serum albumin (BSA), n-sodium dodecyl sulfate (SDS), NTPDase (Apyrase from potato, Grade VII containing the isoenzyme Desirée with low ATPase/ADPase ratio) were purchased from Sigma (St. Louis, MO). BCA protein assay kit was purchased from Thermo Scientific (Rockford, IL). Sodium hydroxide, sodium chloride, potassium chloride, sodium phosphate (monobasic and dibasic), potassium phosphate (monobasic), potassium chloride, sodium bicarbonate, magnesium chloride,

acetone, acetic acid, formaldehyde, ethanol and other reagents were purchased from Fisher Scientific (Fair Lawn, NJ). Platelet rich plasma (PRP) was donated by Dr. Wei Yin, School of Mechanical and Aerospace Engineering, Oklahoma State University. For performing whole blood studies, fresh samples of human blood were drawn from willing donors using buffered sodium citrate as the anti-coagulant.

PET-COOH-NTPDase was prepared using the protocol described in Chapter 3. Briefly, PET was incubated in acetone for 24 h, then reacted with 18.5% formaldehyde solution in 1 M acetic acid for 4 h (Massia and Hubbell 1990) and finally reacted with 1 M bromoacetic acid solution in 2 M sodium hydroxide for 18 h (Löfås and Johnsson 1990) . This formed carboxylated PET (PET-COOH) (Yang *et al.* 2000). PET-COOH was incubated with 0.1 M EDC and 20 U/ml of NTPDase in 0.1 M phosphate buffer (pH = 6.8) at 4˚C for 16 h to form PET-COOH-NTPDase. The following *in vitro* studies were performed on unmodified PET, PET-COOH and PET-COOH-NTPDase to verify haemocompatibility.

5.2.1 Protein adsorption studies

Protein adsorption to the surface of unmodified, carboxylated and NTPDase-modified PET was analyzed using the protocol described in previous studies (Ishihara *et al.* 1999b; Lin *et al.* 2004; Liu *et al.* 2008). Unmodified and modified PET films were equilibrated in phosphate buffered saline (PBS) (pH = 7.4) for 1 h at 37ºC. 1 mg/ml of BSA solution was prepared in PBS. The equilibrated films were then incubated in freshly prepared BSA solution at 37ºC for 3 hours with gentle shaking. The films were washed

gently with PBS to remove weakly adsorbed proteins. The films were then sonicated with 1 wt% n-sodium dodecyl sulfate (SDS) for 1 hour to remove the adsorbed proteins. The protein concentration in the SDS solution was estimated using bicinchoninic acid (BCA) protein assay (Smith *et al.* 1985; Wiechelman *et al.* 1988; Brown *et al.* 1989). First, the BCA working reagent was prepared by mixing 50 ml of reagent A containing bicinchoninic acid, sodium bicarbonate, sodium tartarate and sodium carbonate in 0.1 M sodium hydroxide and 1 ml of reagent B containing 4% cupric sulfate in DI water. 50 µl of sample solution was then mixed with 200 µl of the working reagent. This mixture was incubated at 60ºC for 30 minutes. The absorbance was measured at 560 nm. The assay was calibrated using BSA standards.

5.2.2 Platelet rich plasma (PRP) studies

Platelet adhesion on the surface of unmodified and modified PET was analyzed using a protocol described in literature (Tamada *et al.* 1995; Kang *et al.* 1997; Lee *et al.* 1998; Li and Ruckenstein 2004; Lin *et al.* 2004; Mao *et al.* 2005; Wu *et al.* 2007; Liu *et al.* 2008). Unmodified PET, PET-COOH and PET-COOH-NTPDase films were cut to 1 cm × 1 cm pieces. These films were washed with DI water and placed in 24-well culture plates. PRP from human blood was donated by Dr. Wei Yin. Platelet counts in the platelet rich plasma were 1×10^6 platelets per µl. This PRP was diluted using HEPES-Tyrode's buffer (pH = 7.4) to reduce the platelet count to 350,000 platelets per µl. HEPES-Tyrode's buffer was prepared using the recipe from literature (Lian *et al.* 2005). The buffer contained 134 mM NaCl, 3 mm KCl, 0.3 mM NaH_2PO_4, 12 mM $NaHCO_3$, 2

mM $MgCl_2$, 5 mM HEPES, 5 mM glucose and 0.35% bovine albumin. The diluted PRP was added to each of the wells in the 24-well culture plate containing polymer films. This was incubated at 37ºC for 2 hours without shaking. The films were then washed gently with PBS to remove loosely adhered platelets. The platelets adhered to the polymer surfaces were then fixed with 4% formaldehyde solution in PBS (pH = 7.4) for 1 h at room temperature. The polymer films with adhered platelets were then dehydrated using an ethanol graded series (50, 60, 70, 80, 90 and 100 volume %) for 30 minutes each. The polymers were then dried in a vacuum dessicator at room temperature for 12 hours. Platelet adhesion on unmodified PET, PET-COOH and PET-COOH-NTPDase surfaces was analyzed using SEM images.

5.2.3 Whole blood studies

Thrombus formation on unmodified PET, PET-COOH and PET-COOH-NTPDase surfaces was studied by exposing the polymers to freshly drawn human blood (Kang *et al.* 1997; Lin *et al.* 2004; Liu *et al.* 2008). Human blood was drawn from willing donors using buffered 0.109 M sodium citrate anticoagulant. Although many anticoagulants were available to choose from, sodium citrate was used as it didn't affect normal platelet function. Polymer films were cut to 1 cm × 1 cm pieces and washed with DI water. These pieces were then placed into the wells of a 24-well culture plate. Citrated human blood (0.8 ml) was added to the wells and incubated at 37ºC for 2 hours. The films were then washed gently using PBS to remove any loosely adhered platelets and other cells. The thrombi formed on the surfaces were fixed using 4% formaldehyde solution in PBS (pH

= 7.4) for 1 hour at room temperature. The polymers were then dehydrated using an ethanol graded series (50, 60, 70, 80, 90 and 100 volume %) for 30 minutes each. The polymers were then dried in a vacuum dessicator at room temperature for 12 hours. Thrombi formation on unmodified PET, PET-COOH and PET-COOH-NTPDase surfaces was analyzed using SEM images.

5.3 Results and Discussion

5.3.1 Protein adsorption studies

Protein adsorption is the very first event that occurs at the blood-material interface. Understanding protein-surface interactions has been one of the most important topics in biomaterials and has been explored for several decades. Protein adsorption is known to take place within the first few seconds of exposure to blood (Vroman 1967; Vroman and Adams 1969). It has also been shown that there is layer of protein between the surface and adhered platelets and thrombi (Dutton *et al.* 1969). Therefore, the interaction between blood and the material is mediated by a layer of protein on the surface. A number of studies have shown that protein adsorption on the material surface has a direct effect on platelet adhesion and activation (Kim *et al.* 1974; Lyman *et al.* 1975; Brash and Uniyal 1979; Brash 1991; Tanaka *et al.* 2000; Higuchi *et al.* 2002; Zhang *et al.* 2008). Many researchers have studied the adsorption of one or more of the most abundant plasma proteins, namely, albumin, immunoglobulin G and fibrinogen in buffered solution (Brash and Uniyal 1979; Brash 1991; Lee *et al.* 1997; Zhang *et al.*

2008). These studies also show that adsorption of plasma proteins on the material surface plays a significant role in thrombus formation (Pankowsky *et al.* 1990; Ishihara *et al.* 1991; Fedel *et al.* 2008; Mao *et al.* 2008; Lin *et al.* 2009). Based on these studies, it is clear that minimizing protein adsorption will play a key role in the haemocompatibility of the polymer.

Protein adsorption on the surface depends on a variety of surface properties, such as hydrophilicity, roughness, charge and chemistry. It was important to analyze the influence of carboxylation and NTPDase modification of PET on protein adsorption as increased protein adsorption will increase platelet adhesion and aggregation and result in thrombus formation. For this reason, unmodified PET, PET-COOH and PET-COOH-NTPDase were all incubated with bovine serum albumin, using the protocol described. The concentration of adsorbed albumin was measured using the BCA assay. The results obtained are shown in Figure 5.1.

The results show that unmodified PET and PET-COOH-NTPDase adsorb similar concentrations of albumin. Therefore, attaching NTPDase to PET will not increase platelet activation and the blood coagulation cascade due to protein adsorption. On the other hand, it is seen that PET-COOH adsorbs a higher concentration of albumin. This is probably because of the difference in the surface chemistry. Similar results were observed with arginine modified PET, where aminolyzed PET showed an increase in protein adsorption compared to unmodified PET and arginine attached aminolyzed PET (Liu *et al.* 2008). As NTPDase modification reduced the protein adsorption, further studies were not performed to study the increased protein adsorption on PET-COOH.

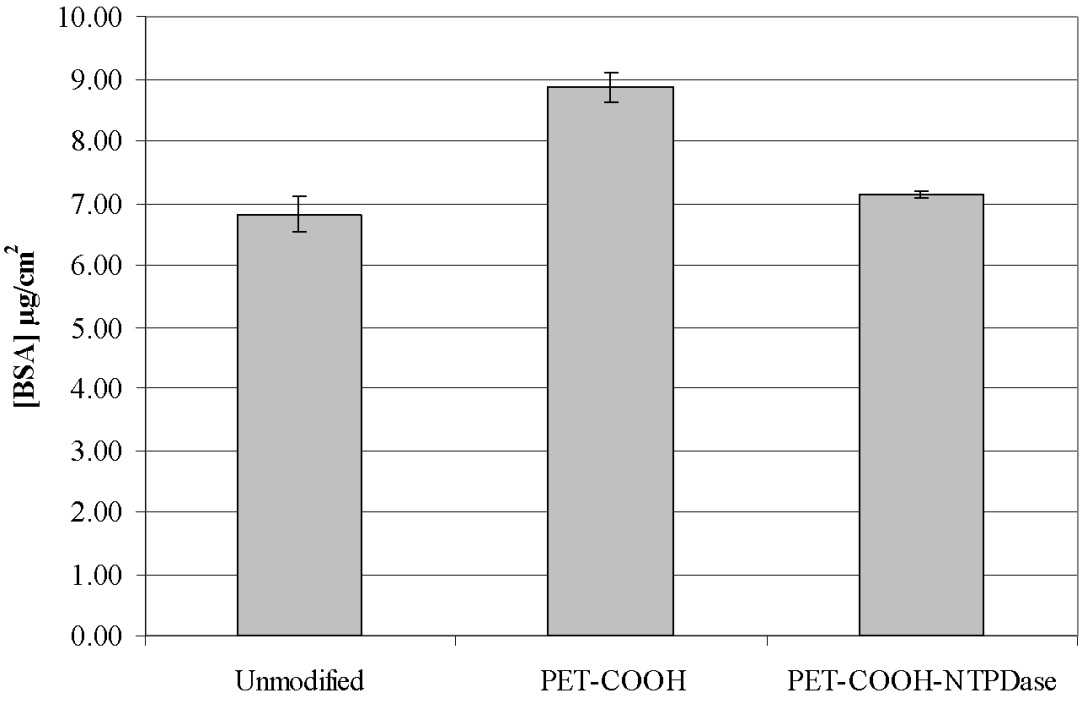


Figure 5.1. Protein adsorption studies on unmodified and modified PET surfaces.

5.3.2 Platelet rich plasma studies

From protein adsorption studies, it is evident that platelet activation caused by adsorbed proteins will be comparable for unmodified PET and PET-COOH-NTPDase. However, PET-COOH-NTPDase showed significant NTPDase activity. Hence, NTPDase will scavenge ADP released during platelet activation, thereby preventing further platelet activation. Therefore, it was expected that minimal platelet activation would be observed on PET-COOH-NTPDase. Based on this hypothesis, it was expected that NTPDase-modified PET will show improved platelet inhibition. For verifying this hypothesis, PRP studies were performed on unmodified and modified PET. *In vitro* studies using PRP is a very common technique used in evaluating haemocompatibility. PET-COOH-NTPDase was exposed to PRP (350,000 platelets per µl) in static conditions at 37ºC for 2 hours. Platelet adhesion on PET-COOH-NTPDase surfaces was then compared with that on unmodified PET and PET-COOH surfaces. This comparison was performed qualitatively using scanning electron microscope (SEM) images. The results obtained are shown in Figures 5.2, 5.3 and 5.4.

Figure 5.2 shows the platelet adhesion on unmodified PET when exposed to PRP. As seen in the SEM images, platelet adhesion on the unmodified PET is evident. The presence of pseudopods on the adhered platelets indicates that the platelets are activated. Based on the SEM images, platelet adhesion on unmodified PET surfaces is probably secondary adhesion. This is an irreversible attachment of platelets to the surface. On close observation, SEM images also indicate platelet aggregation during exposure to platelet rich plasma.

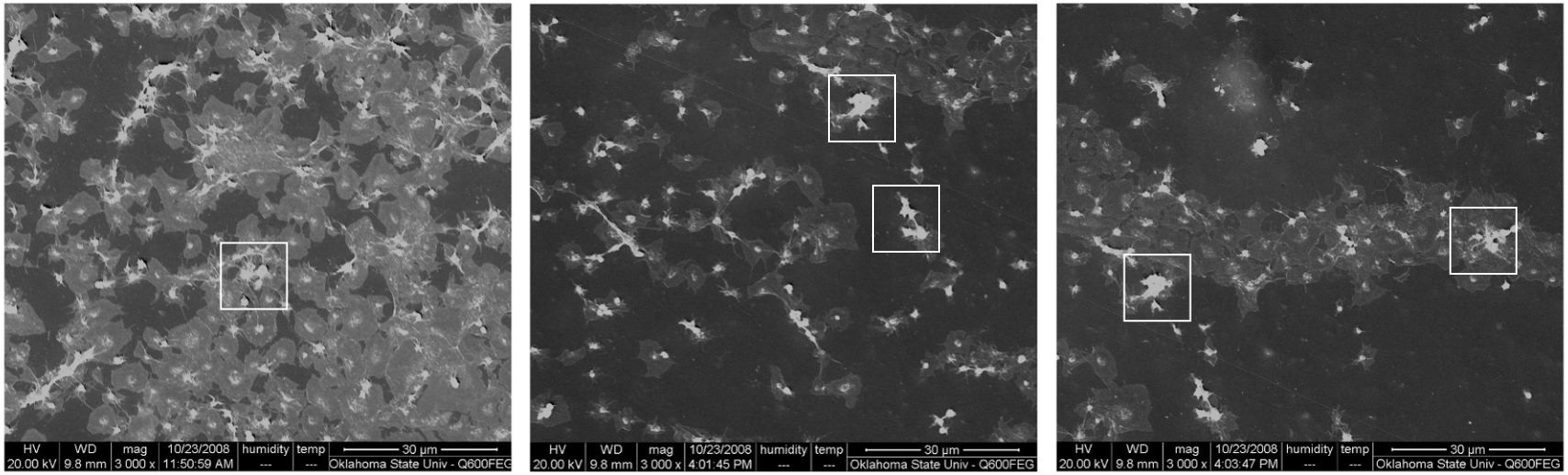


Figure 5.2. SEM images of unmodified PET exposed to platelet rich plasma to understand platelet adhesion. All images are at 3000x magnification. Platelet aggregates are highlighted.

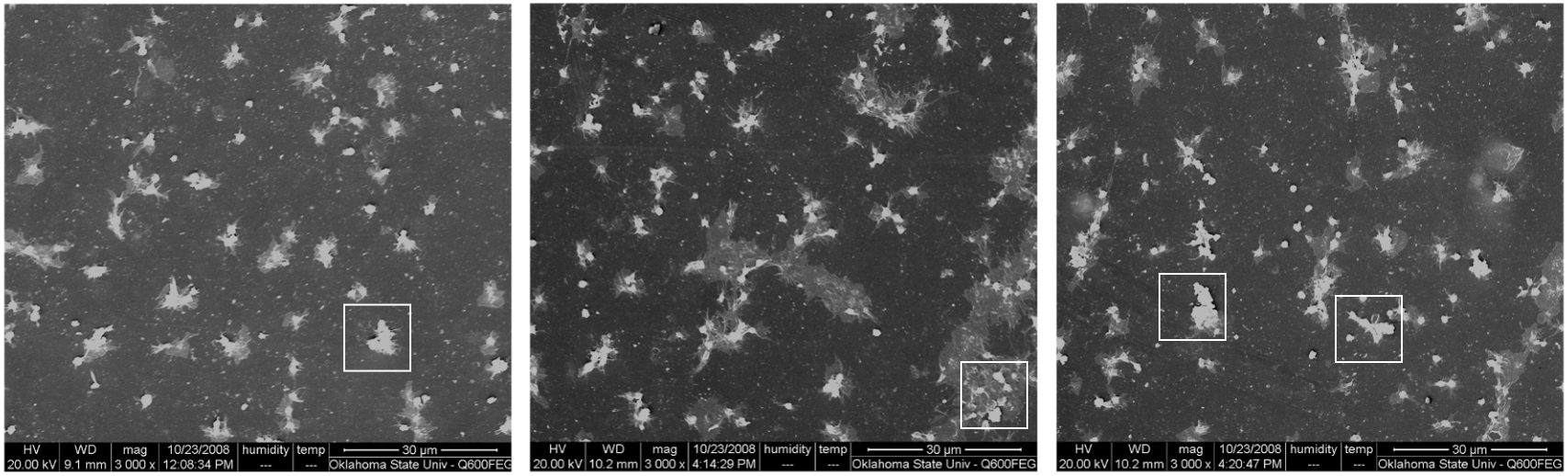


Figure 5.3. SEM images of PET-COOH exposed to platelet rich plasma to understand platelet adhesion. All images are at 3000x magnification. Platelet aggregates are highlighted.

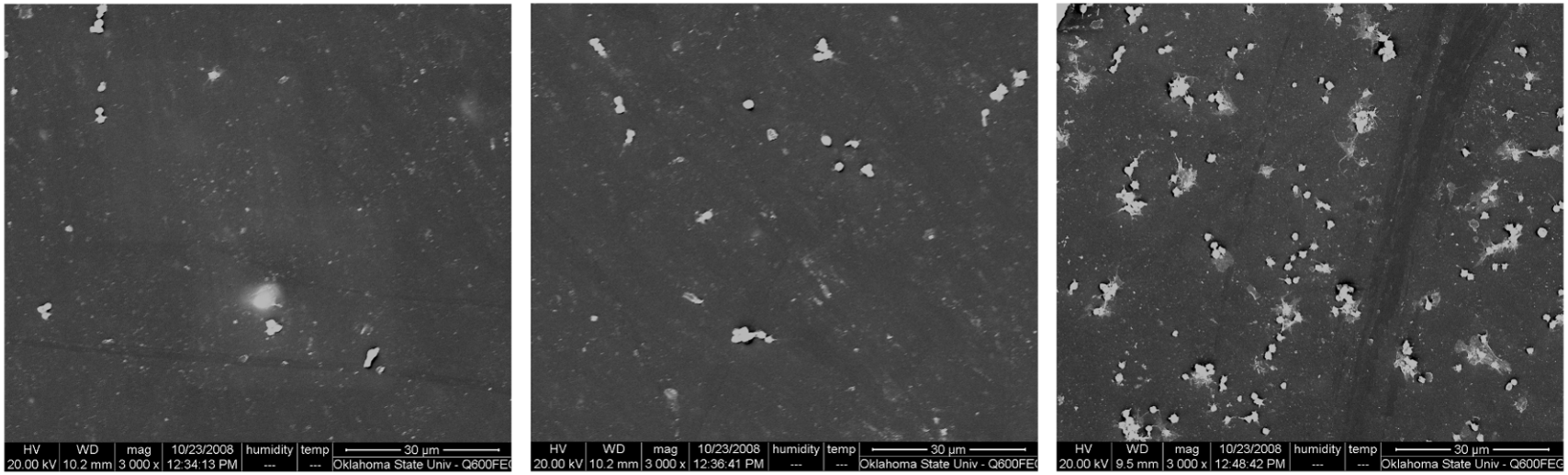


Figure 5.4. SEM images of PET-COOH-NTPDase exposed to platelet rich plasma to understand platelet adhesion. All images are at 3000x magnification.

Figure 5.3 shows the SEM images of carboxylated PET incubated with PRP. The images clearly show significant platelet adhesion on the surface of PET-COOH. Furthermore, from the pseudopods on the platelets adhered, it is evident that platelet activation has occurred. Based on these observations, it is reasonable to conclude that platelet adhesion on the carboxylated PET surface was secondary adhesion. Platelets aggregation is also observed.

Figure 5.4 shows the SEM images of PET-COOH-NTPDase incubated with PRP. NTPDase-modified polymers show inhibition of platelet deposition. In two of the three polymers almost complete platelet inhibition is observed. Very few platelet cells are attached to the surface. Even the platelets attached don't show any visible shape change. However, one of the PET-COOH-NTPDase polymers did show platelet adhesion. But, the platelets are relatively round in shape and didn't exhibit any significant shape change. These observations indicate that platelet activation was reduced by the action of NTPDase. Also, even the platelets adhered to the PET-COOH-NTPDase surface were not aggregated. ADP, a dense granule that induces platelet aggregation, is consumed by the activity of NTPDase. Hence, platelet aggregation is very minimal. These findings indicate that even the reduced platelet adhesion observed on PET-COOH-NTPDase surface is primary adhesion, which is easily reversible.

5.3.3 Whole blood studies

In vitro studies using human blood provides useful information regarding haemocompatibility of a biomaterial. Performing whole blood studies provides

information that is not obtained from PRP studies. When a material is exposed to human blood, first plasma proteins attach to the surface. After this, platelet adhesion and activation occur. Then, the blood coagulation cascade is triggered. This results in thrombus formation. From the protein adsorption and PRP studies, it is expected that thrombus formation would be inhibited on PET-COOH-NTPDase surfaces. For testing this theory, PET-COOH-NTPDase was exposed to freshly drawn citrated human blood at 37ºC for 2 h. Thrombus formation on the PET-COOH-NTPDase surface was analyzed qualitatively using SEM images. These results were compared with the thrombus formation on unmodified and carboxylated PET. The results are shown in Figures 5.5, 5.6 and 5.7.

Figure 5.5 shows the thrombus formation on unmodified PET exposed to human blood. As the SEM images suggest, unmodified PET is highly thrombogenic. This resulted in significant thrombus formation. Also, at higher magnifications, SEM images clearly showed that thrombus formed is strongly adhered to the surfaces. All the unmodified PET surfaces that were exposed to human blood showed significant thrombi formation.

Figure 5.6 shows the thrombus formation on carboxylated PET exposed to human blood. As seen in the SEM images, carboxylated PET also showed significant thrombogenicity. Higher magnification images showed that thrombi on PET-COOH surfaces are also strongly attached. Although significant thrombi formation was observed on carboxylated PET surfaces, thrombi formation was lower than unmodified PET surfaces.

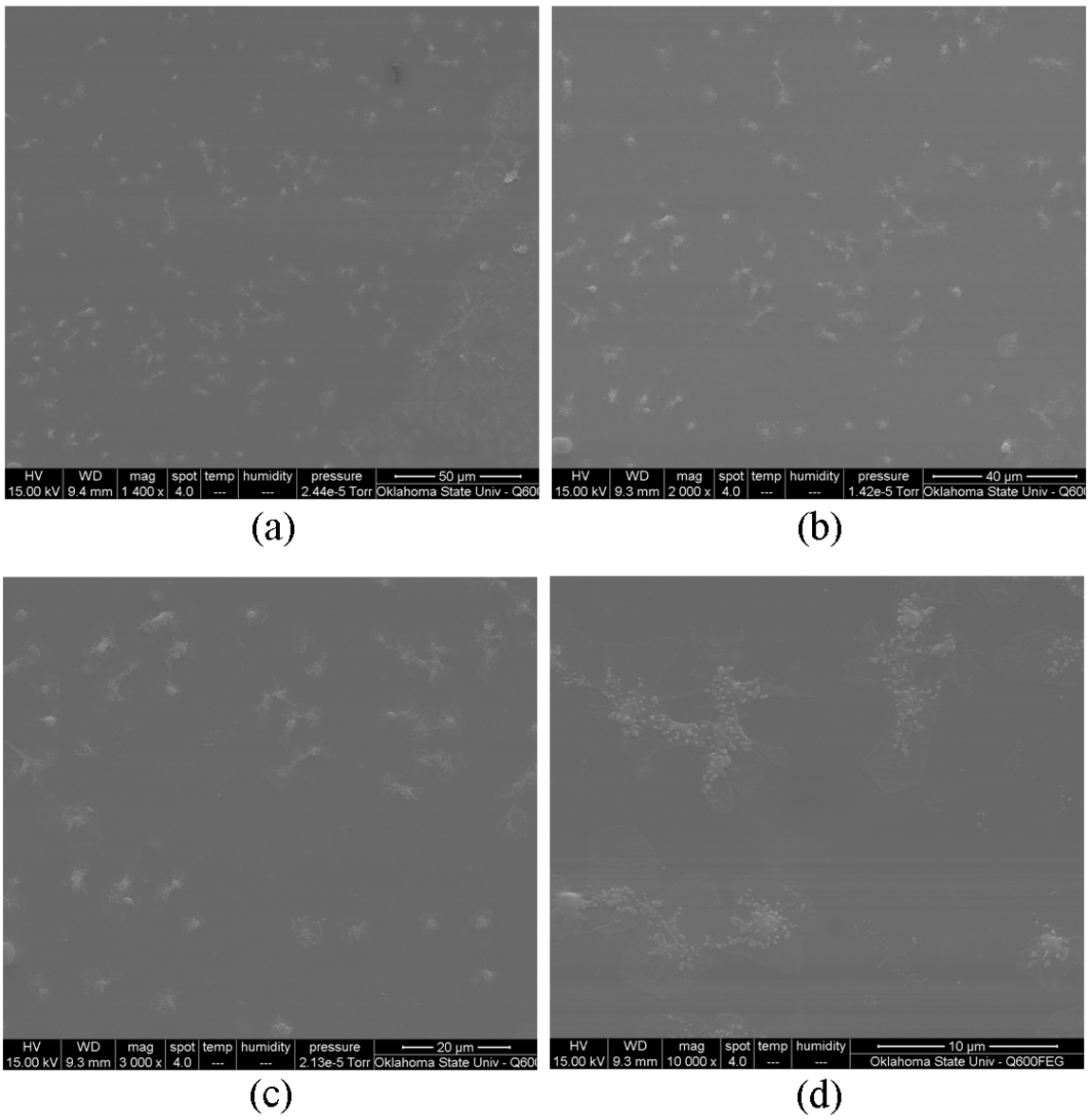
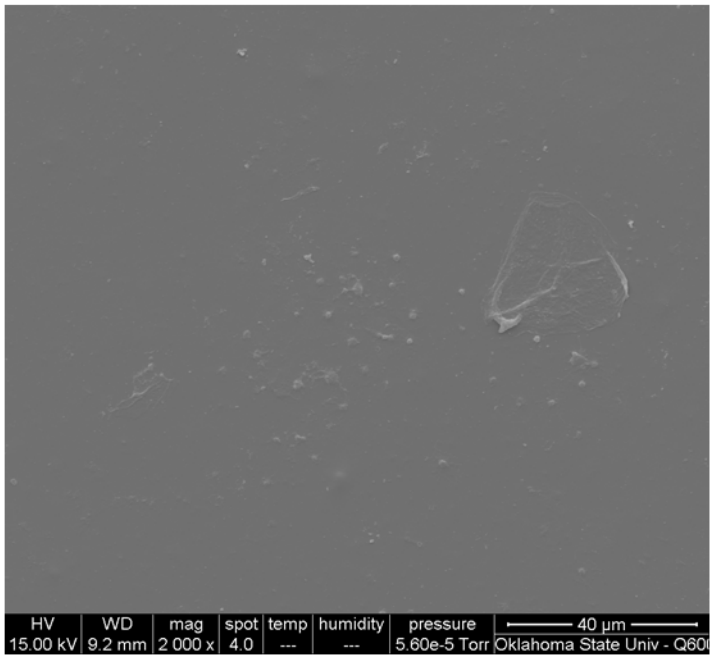
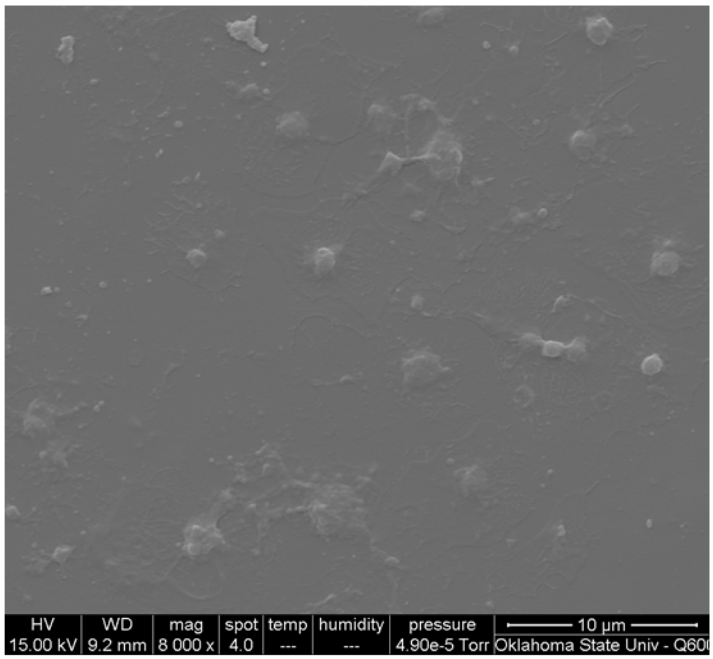


Figure 5.5. SEM images of unmodified PET exposed to whole blood to understand thrombus formation. (a) 1400x, (b) 2000x, (c) 3000x and (d) 10000x magnifications



(a)



(b)

Figure 5.6. SEM images of PET-COOH exposed to whole blood to understand thrombus formation. (a) 2000x and (b) 8000x magnifications

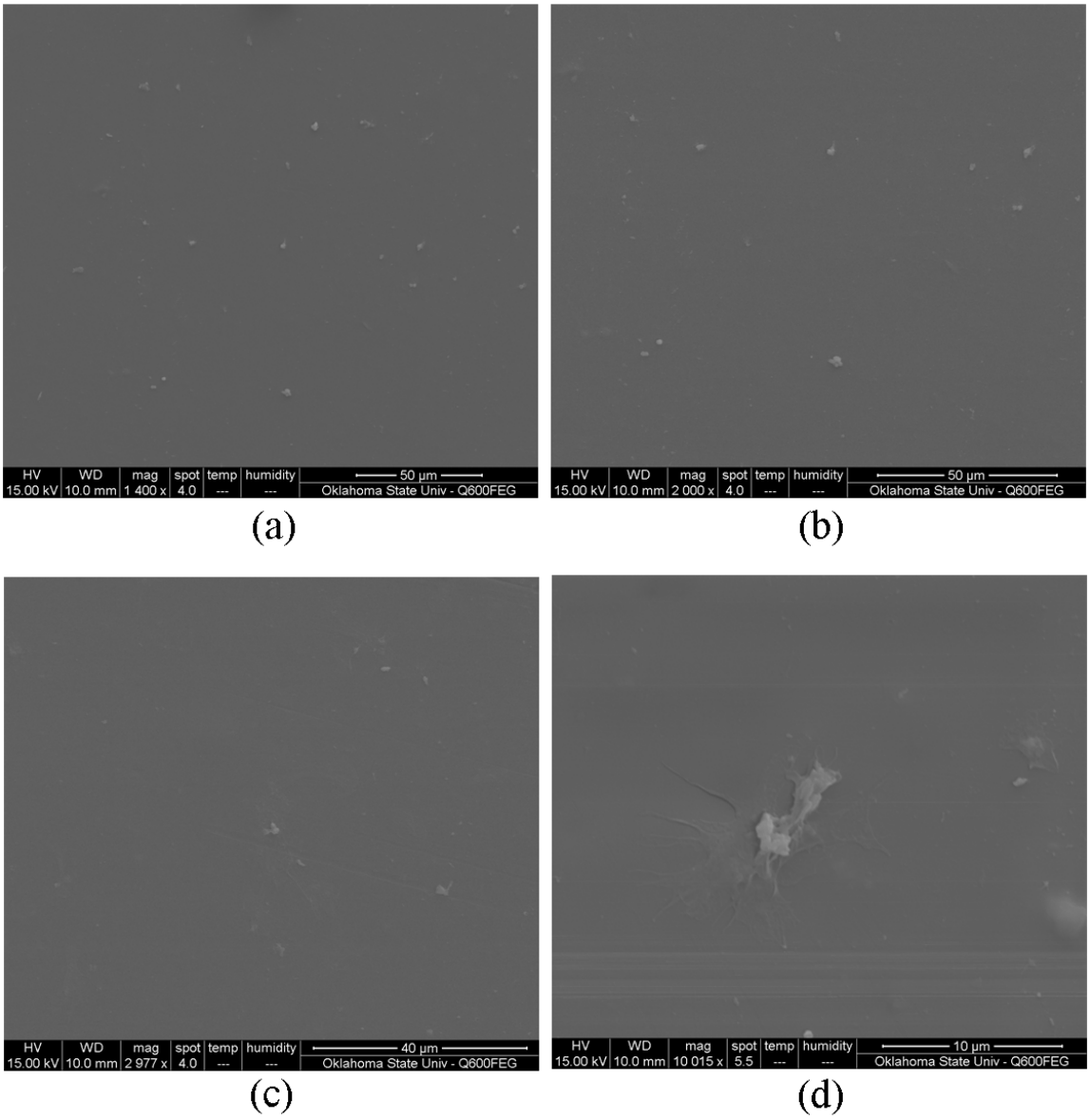


Figure 5.7. SEM images of PET-COOH-NTPDase exposed to whole blood to understand thrombus formation (a) 1400x, (b) 2000x, (c) 2977x and (d) 10000x magnifications

Figure 5.7 shows the thrombus formation on PET-COOH-NTPDase surface exposed to human blood. The SEM images clearly show reduced thrombogenicity. PET-COOH-NTPDase shows very little thrombi formation. Even the few platelets that adhered to the surface did not show any pseudopods, indicating minimal platelet activation. Even under higher magnifications, it is clear that thrombi formation was significantly reduced on PET-COOH-NTPDase. The presence of active NTPDase on PET-COOH-NTPDase clearly enhances anti-thrombogenicity.

5.4 Conclusions

In vitro studies were performed to analyze the haemocompatibility of NTPDase-modified PET. The effect of NTPDase immobilization on protein adsorption, platelet adhesion and thrombus formation were analyzed under static conditions. The results showed that protein adsorption on PET-COOH-NTPDase was comparable to unmodified PET. Also, inhibition of platelet deposition due to NTPDase activity was obvious from the SEM images of unmodified PET, PET-COOH and PET-COOH-NTPDase following exposure to PRP. Whole blood studies showed that thrombus formation is also alleviated by NTPDase activity. On close examination of the SEM images from PRP and whole blood studies, it is apparent that even the platelets adhering to the surface of PET-COOH-NTPDase were not activated. Hence, the platelet adhesion observed on the surface of PET-COOH-NTPDase was probably primary adhesion, which is reversible. Based on these *in vitro* haemocompatibility studies, it is clear that PET-COOH-NTPDase shows enhanced platelet inhibition. As enhanced haemocompatibility is observed only on PET-

COOH-NTPDase and not on unmodified PET and PET-COOH, it is clear that NTPDase activity resulted in ADP scavenging, which in turn inhibits platelet deposition and thrombus formation. With desirable surface and bulk properties of carboxylated PET, improved haemocompatibility due to NTPDase immobilization makes the polymer a viable option in blood-contacting systems.

CHAPTER 6

DUAL FUNCTIONAL POLYMER TO EXPLOIT THE ADDITIVE EFFECT OF NTPDase AND NITRIC OXIDE (NO)

In vitro studies demonstrated that modifying PET surfaces with NTPDase improved the haemocompatibility. In addition to using NTPDase, utilizing another naturally occurring platelet inhibition mechanism that provides an additive effect would potentially cause complete platelet inhibition. One of the molecules known to have an additive effect on platelet inhibition with NTPDase is nitric oxide (NO) (Ramamurthi *et al.* 2001). As nitric oxide is preferentially transferred from S-nitroso albumin to cysteine (Meyer *et al.* 1994), the platelet inhibition effect of nitric oxide has been utilized to improve the haemocompatibility by attaching L-cysteine to polymer surfaces (Duan and Lewis 2002). Attaching L-cysteine, along with NTPDase, to PET would take advantage of the additive effect of NTPDase and NO. This chapter discusses covalent immobilization of L-cysteine and NTPDase to carboxylated PET. The effectiveness of NTPDase attachment was studied by performing NTPDase activity studies. The concentration of L-cysteine immobilized on the dual functional polymer was also measured. Studies were done to verify the possibility of varying the L-cysteine and NTPDase surface concentrations by varying the ratio of L-cysteine to NTPDase during the immobilization process.

6.1 Introduction

For developing a dual functional polymer that makes the most of two naturally occurring platelet inhibition mechanisms, choosing two pathways that have an additive effect on platelet inhibition is critical. In Chapter 5, *in vitro* studies demonstrated that NTPDase-modified PET showed enhanced haemocompatibility. Hence, choosing a mechanism, which works in an additive manner to the platelet inhibition caused by NTPDase, would potentially develop a biomaterial that exhibits total platelet inhibition. NO is a potent platelet inhibitor that attenuates platelet adhesion in multiple ways (Mellion *et al.* 1981; Mendelsohn *et al.* 1990; Davenpek *et al.* 1994; Maurohara *et al.* 1995; Wang *et al.* 1998). NO is known to have an additive effect with NTPDase in platelet inhibition (Ramamurthi *et al.* 2001). Ramamurthi *et al*. showed that NTPDase enhanced the platelet inhibitory effect of NO in a dose-dependent manner *in vitro*, when NTPDase and NO were in solution. Results from this study showed that, for 0.05 ppm NO at 250 s^{-1}, inhibition of platelet deposition increased from 43 ± 5% (no NTPDase) to 62 ± 6% (0.025 U/ml NTPDase) and 89 ± 6% (0.05 U/ml NTPDase) (Ramamurthi *et al.* 2001). Similar results were also observed for other concentrations of NO (Ramamurthi *et al.* 2001). These results clearly showed that NTPDase enhanced the platelet inhibition potency of NO in a dose-dependent manner. The summing of individual platelet inhibition percentages by NTPDase and NO, separately, was equal to the total inhibition produced by NTPDase and NO, proving the additive effect of NTPDase and NO on platelet inhibition (Ramamurthi *et al.* 2001). Based on this, it is expected that NTPDase and NO would function together to improve the haemocompatibility of a biomaterial.

NO-mediated platelet inhibition has been used to improve the haemocompatibility by attaching NO-releasing compounds to a polymer (Saavedra *et al.* 1996; Smith *et al.* 1996). The major limitation of attaching NO-releasing compounds to a polymer is the exhaustion of NO molecules. This limitation can be overcome by utilizing endogenous NO. L-Cysteine can exploit endogenous NO. NO is a highly reactive molecule that is present in the blood stream as S-nitrosothiols, primarily S-nitroso albumin (Stamler *et al.* 1992). However, since S-nitroso albumin can't be transported easily in to the cells, NO must be transferred from S-nitroso albumin to perform physiological activities. Scharfstein *et al*. reported that NO is transferred from S-nitroso albumin and low molecular weight thiols, such as L-cysteine, in both *in vitro* and *in vivo* conditions (Scharfstein *et al.* 1994). NO transfer, under *in vivo* conditions, occurs transiently from high molecular weight thiols to low molecular weight thiols (Scharfstein *et al.* 1994). This reversible transfer of NO from an S-nitrosothiol to a thiol in exchange for a hydrogen atom is defined as transnitrosation (Feelisch *et al.* 1994). The reaction scheme of transnitrosation between S-nitrosoalbumin and L-cysteine can be given as follows.

$$AlbSNO + CySH \rightarrow CySNO + AlbSH \qquad (6.1)$$

Sulfhydryl amino acid residues (RSH), such as L-cysteine, have an affinity to NO up to three orders of magnitude greater than biological compounds (Wink *et al.* 1994). Kinetic studies on transnitrosation between S-nitrosothiols and free thiols, using glutathione, cysteine and serum albumin, showed that NO from S-nitroso albumin transferred rapidly to cysteine (Meyer *et al.* 1994). From a platelet inhibition standpoint,

NO is rapidly released from unstable CySNO, inhibiting platelet adhesion by multiple mechanisms. NO inhibits platelet adhesion by binding to soluble guanylate cyclase and catalyzing the conversion of guanosine triphosphate (GTP) to 3, 5-cyclic guanosine monophosphate (cGMP) (Reynolds and Burstyn 2000), decreasing intracellular levels of Ca^{2+} (Jin *et al.* 2005), inhibiting thromboxane A_2 via the cGMP-dependent protein kinase (Wang *et al.* 1998), attenuating the expression of P-selectin via cGMP-dependent protein kinase (Davenpek *et al.* 1994; Maurohara *et al.* 1995), reducing the number of GPIIb/IIIa receptors on the platelet surface (Mendelsohn *et al.* 1990), and increasing the dissociation constant of the receptor for the fibrinogen (Jin *et al.* 2005). As illustrated in Figure 6.1a, attaching L-cysteine utilizes endogenous NO to activate the platelet inhibition caused by NO and improves the haemocompatibility of PET and polyurethane by up to 67% (Duan and Lewis 2002).

Based on the results from Ramamurthi *et al*. (2001) and Duan and Lewis (2002), it was expected that successful immobilization of L-cysteine and NTPDase to a biomaterial would significantly improve the haemocompatibility of the material. For this reason, developing a dual functional polymer that exploits ADP scavenging by NTPDase and NO-mediated platelet inhibition by L-cysteine (Figure 6.1b) will have a significant impact in the biomedical applications of the polymer.

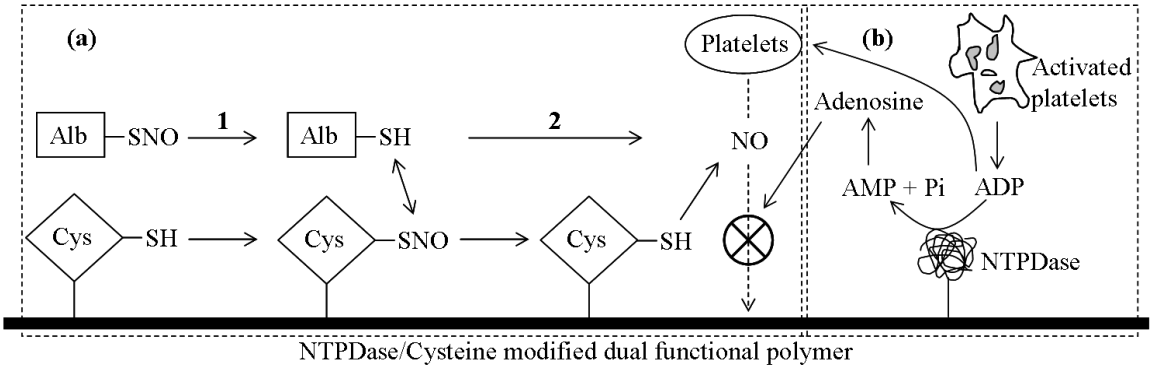


Figure 6.1. Schematic diagram showing the mechanism of exploiting endogenous NO and ADP scavenging using an NTPDase/cysteine-modified dual functional polymer to improve haemocompatibility. (a) L-cysteine utilizing endogenous NO (1) Transnitrosation – NO transfer from S-nitrosoalbumin to L-cysteine immobilized on the surface, (2) Decomposition – Spontaneous release of NO from unstable S-nitrosocysteine. (b) NTPDase scavenging ADP and forming adenosine

6.2 Materials and methods

Polyethylene terephthalate (PET) (thickness = 0.2 mm) was supplied by DuPont (Hopewell, VA). Bromoacetic acid, 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC), L-cysteine, sodium nitrite, ethylenediaminetetraacetic acid, Ellman's reagent, malachite green hydrochloride, ammonium molybdate tetrahydrate, polyoxyethylenesorbitan monolaurate (Tween 20), tris[hydroxymethyl]aminomethane (Trizma base), adenosine diphosphate (ADP) and NTPDase (Apyrase from potato, Grade VII containing the isoenzyme Desirée with low ATPase/ADPase ratio) were purchased from Sigma (St. Louis, MO). Sodium hydroxide, sodium phosphate (monobasic and dibasic), sodium chloride, acetone, acetic acid, formaldehyde, calcium chloride, sulfuric acid and other reagents were purchased from Fisher Scientific (Fair Lawn, NJ).

A dual functional polymer was developed using the carboxylated PET, which is mechanically strong and exhibits desirable biomedical properties. Before attaching cysteine and NTPDase, PET surfaces were carboxylated using the protocol described in Chapter 3 (Yang *et al.* 2000). Briefly, acetone treated PET was hydroxylated by reacting with 18.5% formaldehyde solution in 1 M acetic acid for 4 h (Massia and Hubbell 1990). The hydroxylated PET was then carboxylated by reacting with 1 M bromoacetic acid solution in 2 M sodium hydroxide for 18 h (Löfås and Johnsson 1990) . Carboxylated PET was washed thoroughly with DI water and dried in a glove box under a nitrogen environment before use.

6.2.1 Dual functional polymer

Two batches of NTPDase/cysteine-modified dual functional PET were prepared. For the first batch, carboxylated PET was reacted with 3 ml of 0.1 M EDC, 1.5 ml of 20 U/ml NTPDase and 1.5 ml of 0.1 M cysteine in 0.1 M phosphate buffer (pH = 6.8) at 4˚C for 16 h. This immobilized NTPDase and cysteine on carboxylated PET using the amide bonds. The ε-amine group of the lysine molecules on NTPDase (Marconi *et al.* 1983) and the primary amine group on cysteine react with the carboxyl groups present on the carboxylated PET (Figure 6.2). This polymer is referred to as high NTPDase/low cysteine PET. Another batch was prepared by reacting carboxylated PET with 3 ml of 0.1 M EDC, 1.5 ml of 20 U/ml NTPDase and 1.5 ml of 0.2 M cysteine in 0.1 M phosphate buffer (pH = 6.8) at 4ºC for 16 h. This polymer is referred to as low NTPDase/high cysteine PET. This polymer should have a higher cysteine surface concentration compared to the high NTPDase/low cysteine PET.

6.2.2 Cysteine immobilization

Cysteine was also attached separately to carboxylated PET to study the stability of cysteine immobilization. For immobilizing cysteine to carboxylated PET, the modified polymer was incubated with 0.25 M EDC and 0.25 M cysteine in 0.1 M phosphate buffer (pH = 6.8) at 4˚C for 16 h. This formed an amide between the carboxyl group on the carboxylated PET and the amine group on cysteine. This modified polymer is referred as PET-COOH-Cys.

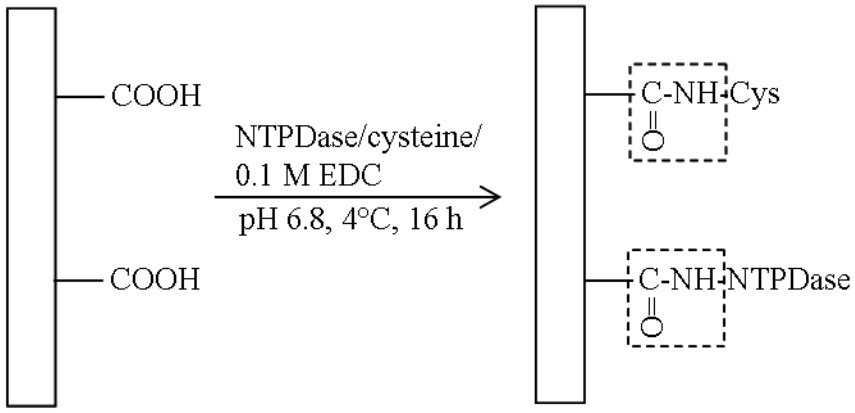


Figure 6.2. Schematic diagram of NTPDase and cysteine immobilization on carboxylated PET to form a dual functional polymer. The amide bonds formed during immobilization are highlighted.

6.2.3 Analysis of NTPDase attachment

The attachment of NTPDase was verified by testing for NTPDase activity on the dual functional polymer. The experimental protocol was similar to the methods described in Chapter 3. Briefly, dual functional polymers were exposed to 50 ml of 0.25 mM ADP in the presence of 0.1 M Tris buffer (pH = 7.4) and 5 mM $CaCl_2$. ADP was converted to AMP and inorganic phosphate (Pi). The sample solution was assayed for Pi concentration using a malachite green solution assay (Baykov *et al.* 1988; Geladopoulos *et al.* 1991).

6.2.4 Analysis of cysteine attachment

Immobilization of cysteine on the dual functional polymer was verified by measuring the cysteine surface concentration. The cysteine surface concentration was quantified using an indirect method. The dual functional PET was incubated with 20 μM $NaNO_2$ and 0.5 N HCl for 30 min. This decreased the nitrite concentration in the solution since the thiols on the surface are nitrosated. Meanwhile, a similar acidic nitrite solution was prepared, without exposure to a polymer, to use as a blank. Samples (20 μl) were taken from both the polymer-exposed solution and the blank solution and the nitrite concentration was measured by a Sievers NO chemiluminescence analyzer NOA 270B (Sievers Medical Instruments, Inc., Boulder, CO) using a potassium iodide/iodine (KI/I_2) reducing solution in which nitrite was decomposed to form NO. The surface cysteine concentration was estimated by the following equation:

$$[CySH]_{surface} = ([NO_2^-]_{blank} - [NO_2^-]_{sample}) \bullet V / A$$

where $[NO_2^-]_{blank}$ is the nitrite concentration of the blank solution, $[NO_2^-]_{sample}$ is the nitrite concentration of the solution containing the modified PET, V is the volume of the solution and A is the area of the polymer film.

In addition to measuring the surface cysteine concentration, the cysteine concentration in solution was measured during some studies using Ellman's method (Ellman 1959; Rootwelt 1967). Briefly, a 100 µL sample solution was mixed with an equal volume of Ellman's reagent (5mM DTNB in 100mM pH 7.2 phosphate buffer and 0.1mM EDTA) in a 96-well microplate (Falcon 353228). After 10 min, the absorbance of the solution was measured at 410 nm using a Packard® SpectraCount™ Microplate Photometer BS10000 (Packard Instrument Co., IL).

6.3 Results and Discussion

6.3.1 NTPDase activity on the dual functional polymer

The presence of NTPDase on the high NTPDase/low cysteine PET and low NTPDase/high cysteine PET was verified by studying the NTPDase activity of modified polymers. Figure 6.3a shows the NTPDase activity of the dual functional polymers. As seen in these results, it is clear that NTPDase activity is significantly higher in high NTPDase/low cysteine PET compared to low NTPDase/high cysteine PET. This possibly indicates that the surface concentration of NTPDase is higher when the ratio of NTPDase to cysteine is higher during immobilization.

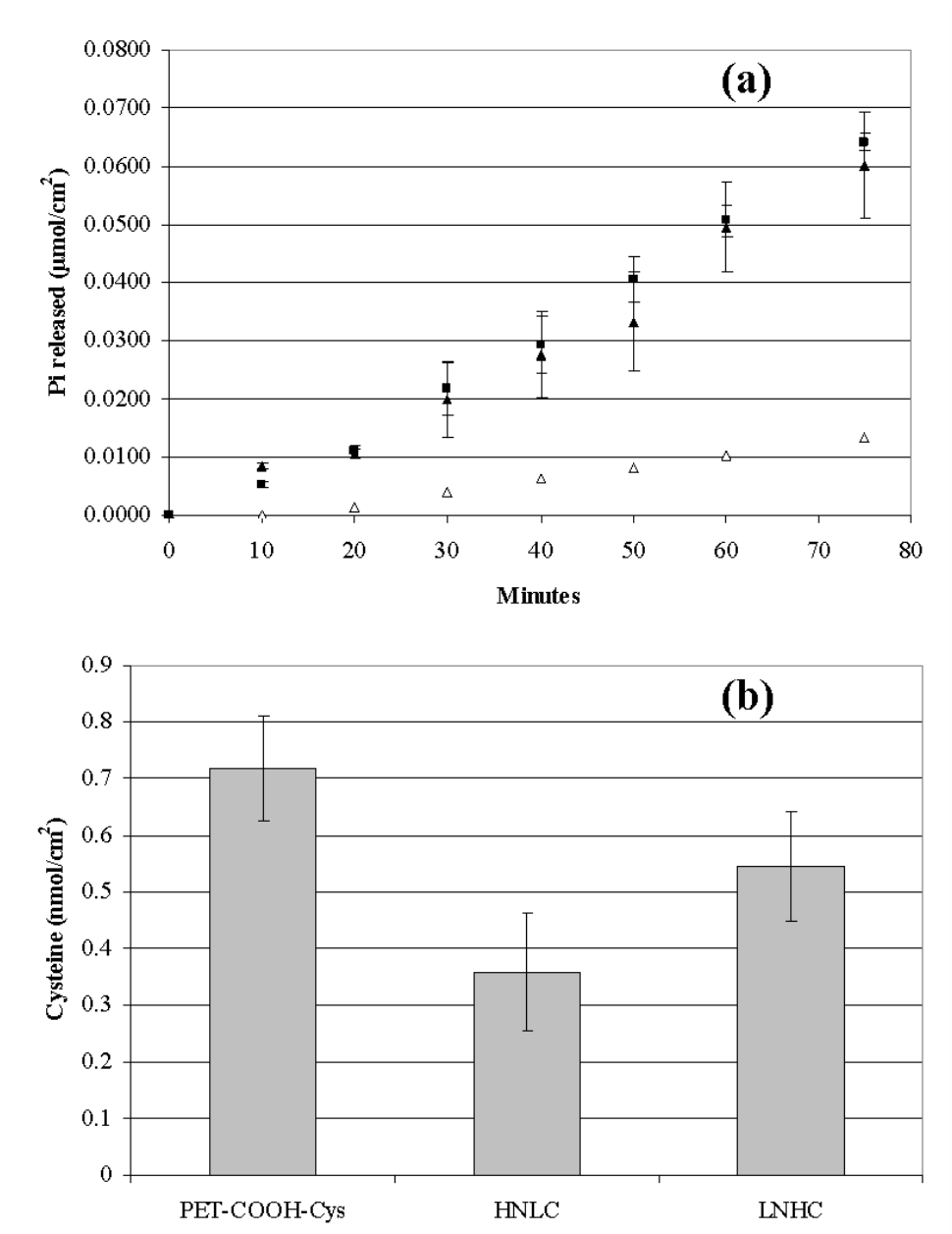


Figure 6.3. Analysis of NTPDase and cysteine attachment on dual functional polymers (a) NTPDase activity on high NTPDase/low cysteine PET (▲), low NTPDase/high cysteine PET (Δ) and PET-COOH-NTPDase (■) (b) Cysteine surface concentrations on PET-COOH-Cys and the dual functional polymers, HNLC – high NTPDase/low cysteine PET, LNHC – low NTPDase/high cysteine PET

Comparing these results with PET-COOH-NTPDase, it is clear that high NTPDase/low cysteine PET had almost similar NTPDase activity. This probably indicates that with low cysteine concentrations, NTPDase immobilization was not altered. Cysteine, a low molecular weight amino acid, probably attached only to the reactive carboxyl groups that were not accessible to NTPDase, a high molecular weight enzyme.

6.3.2 Surface concentration of cysteine on the dual functional polymer

The cysteine surface concentration was measured using chemiluminescence. The results obtained are shown in Figure 6.3b. As seen from the results, it is evident that cysteine is attached to the carboxylated PET. Also, the concentration of cysteine on PET-COOH-Cys and the dual functional polymers were quantifiable. Furthermore, it is clear that the cysteine concentration was higher when the ratio of NTPDase to cysteine used was lower. Therefore, adjusting the ratio of NTPDase to cysteine during carbodiimide coupling will provide different surface concentrations of NTPDase and cysteine. This can be used to optimize the NTPDase and cysteine surface concentrations.

6.3.3 Stability of cysteine immobilization

The stability of the amide bond in PET-COOH-Cys was tested by exposure to water at room temperature for prolonged hours. As seen on Figure 6.4, the cysteine on the surface decreased with time (data obtained from Jun Gu).

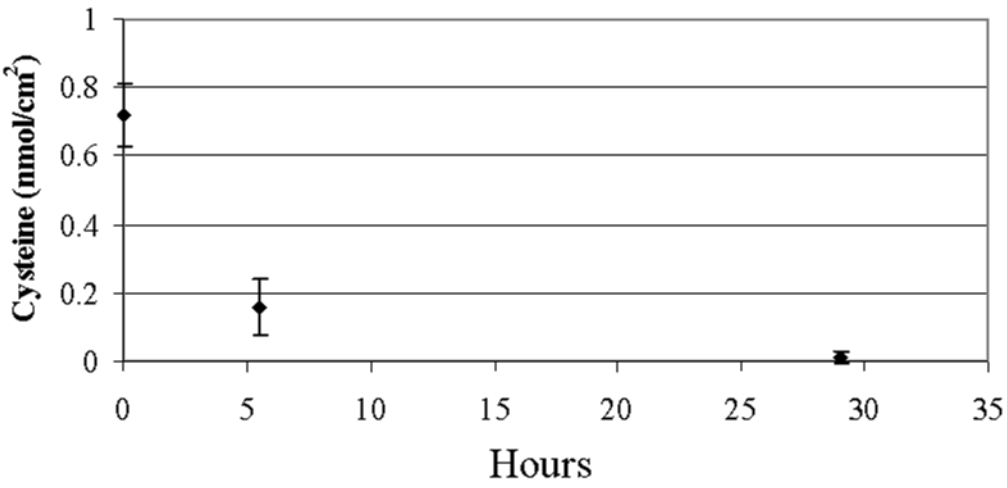


Figure 6.4. Stability of cysteine immobilization on PET-COOH-Cys Cysteine concentrations were measured with time, when stored in DI water. Loss of cysteine from the polymer is probably due to thioester formation during carbodiimide coupling. Data from work done by Jun Gu.

There was detectable (but not reliably quantifiable) amounts of cysteine in solution, indicating cysteine instability as a result of bond breakage. This instability of the amide bond is in contrast to the stable amide bond observed with PET-COOH-NTPDase. The apparent bond breakage may be due to the formation of thioesters instead of amide bonds (Figure 6.5). During carbodiimide coupling, EDC forms a complex with cysteine to activate the carboxyl group. This cysteine with an activated carboxyl group could react with an amine group on another cysteine molecule to form long polymeric chains of cysteine. This would reduce the availability of amine groups on cysteine to react with the carboxyl groups present on the polymer. Therefore, thiol groups on cysteine (rather than amine groups) could react with the activated carboxyl groups on the polymer and form thioester linkages (Hermanson 1996). Formation of such thioester linkages is commonly observed during peptide synthesis when cysteine is used (Hiskey 1980; Bodanszky and Martinez 1983; Atherton *et al.* 1985). These thioester linkages are not as stable as amide bonds in solution (Hermanson 1996). During peptide synthesis, thiol groups are protected to prevent side reactions (Hiskey 1980; Atherton *et al.* 1985; Albericio *et al.* 1989). Using thiol protected cysteine would avoid the formation of thioester linkage during carbodiimide coupling. Thiol groups can later be unprotected by simple hydrolysis. In addition to bond breakage, there is also a possibility of cysteine oxidation.

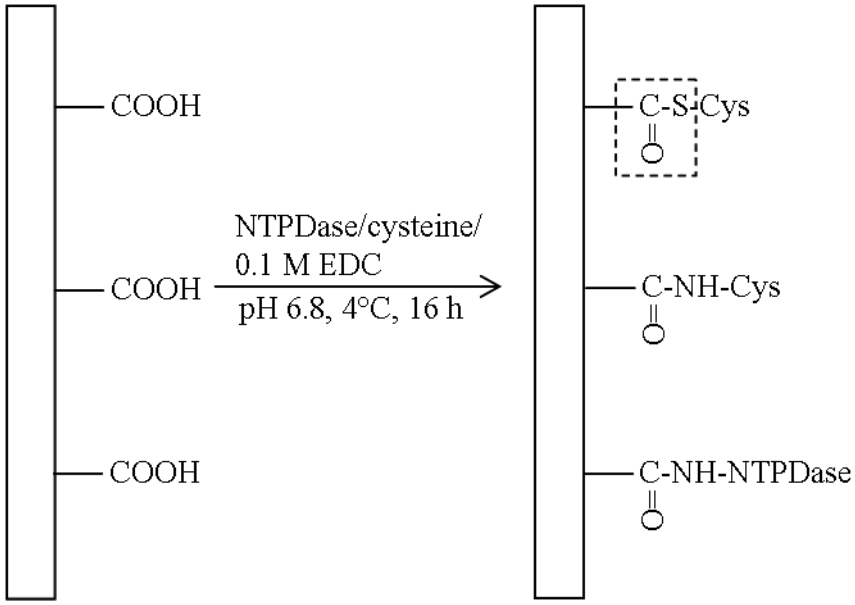


Figure 6.5. Thioester formation in a dual functional polymer. The thioester bond, which might be the reason behind the instability of cysteine immobilization to carboxylated PET, is highlighted.

6.4 Conclusions

A dual functional polymer utilizing NTPDase and NO-mediated platelet inhibition mechanisms would likely show an additive effect on improving haemocompatibility. For this purpose, developing a polymer with stable NTPDase and cysteine immobilization is required. From the studies performed, it is clear that NTPDase and cysteine can be immobilized on carboxylated PET. However, only NTPDase immobilization is stable, while cysteine immobilization is not. This problem is possibly due to the formation of weaker thioester linkages, instead of amide bonds. Based on the peptide synthesis studies, it is expected that this problem can be overcome by using thiol-protected cysteine during immobilization and then unprotecting the thiol group. Although cysteine immobilization was not stable, results for NTPDase activity and initial cysteine surface concentrations indicated that surface distribution of cysteine and NTPDase depends on the ratio of concentrations of NTPDase to cysteine during immobilization. This is valuable information for developing a dual functional polymer with optimal cysteine and NTPDase surface concentration for maximum haemocompatibility.

CHAPTER 7

CONCLUSIONS AND FUTURE WORK

The goal of this research was to develop a haemocompatible polymer, with desirable mechanical and surface properties, by successfully immobilizing NTPDase. The motivation of the project was based on the wide range of biomedical applications in which materials that lack haemocompatibility come in contact with blood. Improving the haemocompatibility of these materials will significantly improve the clinical applications and the quality of life of patients. Successful development of a haemocompatible material with desired biomedical properties will also have a significant economic impact.

Previous studies have shown that NTPDase, when coated or covalently immobilized, can enhance the haemocompatibility of the material (Marconi *et al.* 1983; Bakker *et al.* 1991; van der Lei *et al.* 1992). However, these studies suffered from some serious limitations. NTPDase coating resulted in loss of NTPDase in the blood stream, causing the polymer to lose its haemocompatibility. On the other hand, surface modifications performed to covalently bind NTPDase caused significant changes to the surface and bulk properties of the material. The aim of this research was to overcome these limitations by covalently immobilizing NTPDase on a modified polymer that showed desirable mechanical and surface properties. This mechanically stable NTPDase-modified polymer will scavenge ADP released from activated platelets and inhibit platelet deposition.

7.1 Successfully accomplished objectives

For accomplishing the defined goal, polyethylene terephthalate (PET), a model polymer, was chosen to be modified by attaching NTPDase. PET is used in a wide range of biomedical applications, where it comes in contact with blood. Attaching NTPDase to PET will significantly improve its applications. However, for covalently immobilizing NTPDase to PET, reactive functional groups had to be added to the PET surface. After extensive literature survey, multiple surface functionalization techniques were identified. Out of the different methods, three were specifically chosen for this research. They were aminolysis, hydrolysis and carboxylation.

Aminolysis was an intriguing technique for introducing reactive functional groups to the PET surface because it had been used earlier in this lab for immobilizing cysteine (Duan and Lewis 2002; Gappa-Fahlenkamp and Lewis 2005). Hydrolyzed PET had also been used to immobilize NTPDase by an earlier study (Marconi *et al.* 1983). However, these two techniques have been known to damage to the surface properties and the mechanical strength of PET (Fukatsu 1992; Bùi *et al.* 1993; Kao *et al.* 1998; Liu *et al.* 2005; Nissen *et al.* 2008). Carboxylation of PET was a relatively new method for introducing reactive functional groups to the PET surface (Yang *et al.* 2000; Hyun *et al.* 2001). However, the effect of carboxylation on the surface and bulk properties of PET were not studied. In this research, surface and bulk properties of carboxylated PET were analyzed. The results obtained were compared with aminolyzed PET, hydrolyzed PET and unmodified PET. SEM, AFM and tensile analysis clearly showed that carboxylated

PET showed similar surface and bulk properties as the unmodified PET. These findings clearly show that carboxylation is preferred over aminolysis and hydrolysis of PET.

In this research, NTPDase was successfully immobilized on carboxylated PET (PET-COOH-NTPDase). NTPDase was also immobilized on aminolyzed PET (PET-NH_2-NTPDase). However, the results showed that NTPDase immobilization on aminolyzed PET is not stable. This is potentially due to imine bond breakage. Although imine bonds can be stabilized by reducing them, further investigation was not warranted as the polymer showed significant surface deterioration and loss of mechanical strength. NTPDase immobilized on both carboxylated PET and aminolyzed PET was active. The presence of NTPDase was verified by testing the modified polymers for NTPDase activity. Although PET-NH_2-NTPDase showed much higher NTPDase activity, the polymer can't be used in biomedical applications because (1) NTPDase attachment was not stable, (2) the polymer lost its mechanical strength and (3) significant surface deterioration occurred. NTPDase activity on PET-COOH-NTPDase was lower than the activity on PET-NH_2-NTPDase. However, NTPDase activity on PET-COOH-NTPDase was comparable to the activities observed with NTPDase-modified nylon and C 50 steel rings that showed improved haemocompatibility (Marconi *et al.* 1983). PET-COOH-NTPDase, with quantifiable NTPDase activity and advantageous surface and bulk properties, will be a good biomedical polymer showing improved haemocompatibility.

For understanding the effectiveness of NTPDase immobilization, kinetic studies were performed using PET-NH_2-NTPDase and PET-COOH-NTPDase. Free NTPDase kinetics were also performed for comparison. The results obtained showed that free NTPDase lost activity in solution at 37ºC. However, PET-COOH-NTPDase didn't lose

activity in solution at 37ºC. These results show that immobilizing NTPDase on carboxylated PET improved the stability of NTPDase activity in solution at 37ºC. This could potentially have significant implications for long-term biomedical applications. Furthermore, results obtained from 1.5-ml and 50-ml reaction systems showed that mass transfer limitations play a role in larger volume reactions, while small volume reactions were likely kinetic limited. Immobilized NTPDase kinetics was analyzed using PET-COOH-NTPDase polymers. Although Michaelis-Menten model fit the data, there were inconsistencies with the values of v_{max} and K_M. This was probably due to the limitations with the sensitivity of measuring small changes in Pi concentrations. Also, storing PET-COOH-NTPDase at -20°C could have resulted in some of these inconsistencies. Therefore, with the information currently available, estimation of Michaelis-Menten constants for immobilized NTPDase kinetics is ambiguous. However, all the experiments performed do show that PET-COOH-NTPDase was consistently showing significant NTPDase activity.

For the polymer to be useful in blood-contacting systems, the material should be haemocompatible. To verify this, *in vitro* studies were performed using PET-COOH-NTPDase. As protein-adsorbed surfaces activate platelets and associated thrombus formation (Hanson 2004), the amount of protein adsorbed to the surface of unmodified, carboxylated and NTPDase-modified PET was measured. These protein adsorption studies showed that carboxylation of PET increased the quantity of protein adsorbed to the surface. However, when NTPDase was attached to the carboxylated PET, protein adsorption was comparable to the unmodified PET. Hence, PET-COOH-NTPDase should not cause any platelet activation due to excessive protein adsorption. Platelet rich plasma

and whole blood studies were also performed to verify the extent of clot formation on the polymer surface. SEM images of unmodified, carboxylated and NTPDase-modified PET showed that platelet adhesion was drastically lowered by the presence of NTPDase. Results also show that even the platelets adhered to the surface of PET-COOH-NTPDase were not activated, indicating primary adhesion.

Another objective of this research was to utilize more than one naturally occurring platelet inhibition pathway to improve haemocompatibility. In this work, NO-mediated platelet inhibition, which is known to have an additive effect to NTPDase (Ramamurthi *et al.* 2001), was chosen as the additional pathway. To take advantage of NO-mediated platelet inhibition, cysteine was attached to carboxylated PET along with NTPDase. Results showed that cysteine and NTPDase can both be attached to carboxylated PET. However, only NTPDase attachment was stable. Stability studies performed using PET-COOH-Cys showed that cysteine attachment was not stable in solution. This is possibly due to the formation of weaker thioester bonds, instead of the stable amide bonds. This limitation can be overcome by using thiol-protected cysteine during carbodiimide coupling. The thiol group would later have to be unprotected. Although cysteine attachment wasn't stable, results show that the surface concentration of cysteine and NTPDase depended on the ratio of NTPDase to cysteine used during carbodiimide coupling. Therefore, combined cysteine and NTPDase immobilization can be used to optimize a dual functional polymer to attain increased haemocompatibility.

7.2 Future direction

The findings of this research have contributed significantly to previous results. Further studies can be performed to pursue the ultimate goal of total platelet inhibition.

- Haemocompatibility studies can be performed on PET-COOH-NTPDase under flow conditions. The shear stress caused during blood flow results in the release of ADP, which can cause blood coagulation. Testing PET-COOH-NTPDase with flowing blood will provide further insights for applications of the polymer.
- *Ex vivo* and *in vivo* testing of PET-COOH-NTPDase will provide valuable information that wasn't considered during *in vitro* studies.
- Durability and the half-life of NTPDase activity on PET-COOH-NTPDase will have to be studied for understanding the potential of this polymer in long-term applications, like implants.
- Optimization of NTPDase concentration and activity on PET-COOH-NTPDase will help in further enhancing the haemocompatibility of the polymer.

Developing an effective dual functional polymer that takes advantage of ADP scavenging by NTPDase and NO-mediated platelet inhibition by cysteine will be another useful future direction. For accomplishing this, the following objectives have to be fulfilled.

- Cysteine attachment to carboxylated PET has to be stabilized. For this to occur, thiol-protected cysteine can be used. The thiol group has to be

unprotected later on by simple hydrolysis. However, the technique used must be evaluated with regards to NTPDase activity.

- Once cysteine is successfully immobilized to carboxylated PET, a stable dual functional polymer can be developed.
- Haemocompatibility studies using the dual functional polymer should be done to understand the additive effect of NTPDase and cysteine.
- The surface concentrations of NTPDase and cysteine on the dual functional polymer have to be optimized by using different NTPDase to cysteine ratios during carbodiimide coupling.
- The long-term properties of the dual functional polymer have to be studied.

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IRB Approval Form

Oklahoma State University Institutional Review Board

Date	Wednesday, October 22, 2008	**Protocol Expires:**	**10/21/2009**
IRB Application No:	EG051		
Proposal Title:	Enhanced Biomaterials for Inhibiting Platelet Deposition		
Reviewed and Processed as:	Expedited **Continuation**		

Status Recommended by Reviewer(s): **Approved**

Principal Investigator(s) :

James Smay
423 Engineering North
Stillwater, OK 74078

Approvals are valid for one calendar year, after which time a request for continuation must be submitted. Any modifications to the research project approved by the IRB must be submitted for approval with the advisor's signature. The IRB office MUST be notified in writing when a project is complete. Approved projects are subject to monitoring by the IRB. Expedited and exempt projects may be reviewed by the full Institutional Review Board.

The final versions of any printed recruitment, consent and assent documents bearing the IRB approval stamp are attached to this letter. These are the versions that must be used during the study.

Signature:

Sheila Kennison, Chair, Institutional Review Board

Wednesday, October 22, 2008
Date

VITA

Vignesh Muthuvijayan

Candidate for the Degree of

Doctor of Philosophy

Dissertation: DEVELOPMENT, CHARACTERIZATION AND EVALUATION OF NTPDase-MODIFIED POLYMER FOR INHIBITING PLATELET DEPOSITION

Major Field: Chemical Engineering

Biographical:

Personal Data: Born in Madurai, Tamil Nadu, India, on August 24, 1981, the son of T. Muthuvijayan and M. Niruba Rani

Education: Graduated from Jaigopal Garodia Vivekananda Vidyalaya, Chennai, India in March 1998; received a Bachelor of Technology degree in Chemical Engineering from Anna University, Chennai, India in May 2002; Completed Master of Science in Chemical and Biochemical Engineering at University of Maryland, Baltimore County, Baltimore, Maryland in August, 2004; Completed the requirements for the Doctor of Philosophy in Chemical Engineering at Oklahoma State University, Stillwater, Oklahoma in May 2009.

Experience: Research Assistant, School of Chemical Engineering, Oklahoma State University, August 2004 to Present; Teaching Assistant, School of Chemical Engineering, Oklahoma State University, August 2007 to Present; Research Assistant, Department of Chemical and Biochemical Engineering, University of Maryland, Baltimore County, May 2003 to August 2004; Technical Intern, Fourrts [India] Pharmaceutical Ltd., May 2001 to August 2001; Technical Intern, Manali Petrochemical Ltd., May 2000 to August 2000.

Professional Memberships: American Institute of Chemical Engineers; Omega Chi Epsilon, The National Honors Society for Chemical Engineers.

Name: Vignesh Muthuvijayan Date of Degree: May 2009

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: DEVELOPMENT, CHARACTERIZATION AND EVALUATION OF NTPDase-MODIFIED POLYMER FOR INHIBITING PLATELET DEPOSITION

Pages in Study: 188 Candidate for the Degree of Doctor of Philosophy

Major Field: Chemical Engineering

Scope and Method of Study: The lack of haemocompatibility in biomaterials is a major concern in blood contacting devices. It causes serious complications such as platelet adhesion and aggregation, thrombosis and shedding of emboli, the latter which could lead to death. According to FDA reports, at least 1510 cases of thrombosis were reported in the year 2008 of which 299 of these cases resulted in death. Improving the haemocompatibility of biomaterials will have a significant impact in lowering these numbers. Furthermore, the market for biomaterials exceeds $100 billion. Improving the functionality of a biomaterial will also have a significant economic impact. Therefore, this research focused on improving the haemocompatibility by utilizing naturally occurring platelet inhibition pathways. NTPDase is a naturally occurring enzyme that rapidly metabolizes ADP to regulate platelet adhesion and aggregation. In this research, polyethylene terephthalate (PET), a model polymer with many blood-contact applications, was modified by covalently attaching NTPDase. The surface and bulk properties of the modified PET were analyzed to verify the potential as a biomedical polymer. Cysteine was also immobilized to PET, along with NTPDase, to take advantage of nitric oxide-mediated platelet inhibition, which has an additive effect on platelet inhibition caused by NTPDase.

Findings and Conclusions: NTPDase was successfully immobilized to functionalized PET surfaces. PET surfaces were functionalized by aminolysis, hydrolysis and carboxylation to introduce reactive functional groups. Surface and bulk characterization showed that carboxylated PET has desirable mechanical and surface properties. NTPDase was immobilized to carboxylated and aminolyzed PET. NTPDase immobilization on carboxylated PET was stable and the polymer showed quantifiable NTPDase activity. NTPDase kinetics were studied for free and immobilized NTPDase. *In vitro* studies using unmodified and modified PET showed that NTPDase attachment significantly improved the haemocompatibility of the polymer. Dual functional polymers were developed by attaching cysteine and NTPDase, simultaneously. However, further research is required to stabilize cysteine attachment.

ADVISER'S APPROVAL: Dr. Randy S. Lewis